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ACTION OF ANTIHISTAMINES AND TRANQUILIZERS ON THE CONVERSION OF FIBRINOGEN TO FIBRIN[1]

LÁSZLÓ KÁTÓ AND BÉLA GÖZSY

Abstract

Antihistamines and tranquilizers with antihistaminic effect interfere in vitro with the conversion of fibrinogen to fibrin in the presence of thrombin. Clot opacity measurements of such systems show that the presence of moderate amounts of these reagents permits the formation of polymers, which exhibit low opacity at the time of clotting and are characterized as "fine clot". The reagent, which prolonged clotting time, also produced gels of high rigidity and slight tendency to syneresis. Evidence is presented to show that the compounds act upon fibrinogen rather than on thrombin and that the effects are reversible. The effects are assumed to be the results of interference with van der Waals' forces.

Introduction

It has been reported that antihistamines enhance experimental tuberculosis in guinea pigs and mice. This effect was attributed to the inhibition by the synthetic antihistaminics of the defence processes against infection or of the histaminic effects (1). In later experiments it was found that antihistamines strongly inhibit phagocytosis of tubercle bacilli by guinea pig macrophages (2,3), an effect which is evident in the absence of histamine. Investigations into vascular changes in the skin of rats after local and systemic histamine-depletion showed that four structurally different antihistamines inhibited the India-ink and trypan-blue reactions both in the normal and the depleted rats (4,5). These observations suggest a direct effect of the antihistamines on cells of the reticuloendothelial system (RES) (6). It seemed, however, that it did not exclude the possibility that antihistamines interfere with phagocytic activity of specific cells or with functions of capillary endothelium by reacting with tissue or plasma constituents, thus permitting the formation of molecules which might prevent phagocytosis. We observed earlier that, if antihistamines were added to a peritoneal exudate, no clotting occurred even in the absence of heparin, suggesting an interference with fibrinogen polymerization. Investigations into this phenomenon are reported herewith.

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Contribution from the Institute of Microbiology and Hygiene of the University of Montreal, Canada. Supported by grants from the Ministry of Health of the Province of Quebec (Federal–Provincial Health Research Grants).

Materials and Methods

The action of the compounds tested was studied in vitro on the clotting system of rabbit blood. Reagents were added in increasing amounts from 0.5 to 200 μg/ml to the blood plasma and clotting time was measured by the Lee-White method (7) and recalcification time by the method of Biggs (7). Prothrombin activity and prothrombin time were measured by a modification of Quick's (8) method. Antithromboplastic activity was measured as described by Tocantins (9).

Bovine fibrinogen was used throughout the experiments. Fraction I was a commercial preparation that contained 78% fibrinogen as determined by the method of Morrison (10). Citrate was removed from this powder by dissolving it in 0.45 *M* sodium chloride solution and dialyzing against large volumes of the same solvent for 24 hours with several changes. After filtration the stock solution was assayed by the Morrison method. A second but purified fibrinogen preparation (Behring), containing 84% clottable proteins but no citrate, was similarly used.

The antihistamines and tranquilizers, listed in Table I were kindly donated by the manufacturers. The compounds were of reagent grade and they were used without further purification.

All reagents were dissolved in a sodium chloride – phosphate buffered solution of ionic strength 0.45, of which 0.40 was contributed by sodium chloride and 0.05 by phosphate buffer, at pH 6.3. Samples were tested in a total volume of 2 ml, each containing 2.5 g/liter fibrinogen (unless stated otherwise), adequate amounts of the reagents and 1 unit/ml of thrombin (Frosst) in sodium chloride – phosphate buffered solution. Test tubes of 1 cm in diameter were used. The system without the reagents usually gave a clotting time of 12 minutes. If clotting time was shorter than 12 minutes, the experiments were disregarded or the thrombin solution was adjusted to give the standard 12-minute clotting time. In every series of samples, the pH was tested at the highest concentration of each reagent but it did not vary by more than ± 0.1 from the control sample. Samples were allowed to clot in a water bath at 23–25° C. Clotting time was registered at the time when the necessary rigidity developed. Opacity at different time intervals before and after clotting was measured in samples prepared simultaneously. Measurements were taken at 6000 Å with the Beckman spectrophotometer or the Beckman DK recording spectrophotometer. The methods of Ferry and Shulman (11) were used in order to demonstrate whether the reagents studied cause any irreversible changes in fibrinogen or thrombin.

Results

(1) In Vitro Effect of Reagents on the Clotting of Rabbit Plasma

The compounds listed in Table I had no effect in vitro on the recalcification time, prothrombin time, prothrombin activity, and antithromboplastic activity of rabbit plasma, in concentration ranging from 0.5 to 200 μg/ml. The antihistamines had a slight prolonging effect on the clotting time of rabbit plasma.

(2) Effect of Reagents on the Clotting Time of Fibrinogen

Preliminary experiments showed that the reagents did not alter the pH of the samples by more than ± 0.1 in a concentration of 0.4 g/liter. The ionic strength of the samples is increased maximally by 0.004 in the systems containing 0.4 g/liter of the reagents. These minimal changes in pH and ionic strength brought about by the reagents in the buffered system do not cause an increase or decrease of clotting time or clot opacity. The unadjusted pH and ionic strength was used therefore throughout the experiments. All results reported here were obtained with the powder containing 84% fibrinogen. The clot opacity in the samples (0.25% fibrinogen) containing no reagents was 0.022 at clotting time and was adequately reproducible in successive experiments.

The antihistamines tested prolonged the clotting time (tc). Figure 1 shows logtc plotted against concentration (c) of the tested antihistamines. The initial straight lines of the graphs are recorded as $d\log tc/dc$ in Table I. In every case logtc was a linear function of c, hence the obtained data are comparable. The tested antihistamines do not differ greatly in molecular weight and for this reason the calculation of $d\log tc/dm$ (m = concentration in moles/liter) was omitted. The graphs beyond the linear range representing the prolonged clotting time at a higher concentration of reagents were not taken as measures of the effectiveness. In general the tranquilizers tested had no effect on clotting time in this system, except those which exhibit antihistaminic action. In addition two tranquilizers, chlorpromazine and promazine, both with antihistaminic effect, promptly precipitate the fibrinogen solution.

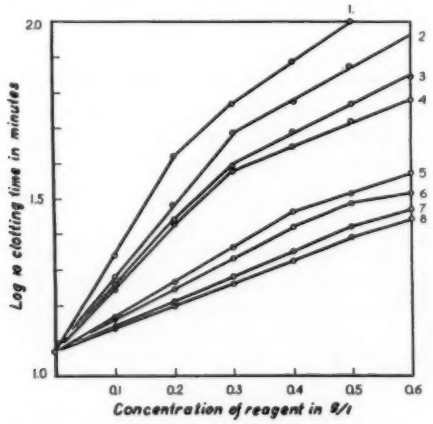

FIG. 1. Logarithm of clotting time in minutes plotted against concentration of antihistamines in g/liter: (1) prophenpyridamine maleate; (2) methyl-N-phenyl-N-thenyl-piperidine tartrate; (3) chlorprophenpyridamine maleate; (4) mepyramine maleate; (5) promethazine HCl; (6) chlorzyclizine HCl; (7) diphenhydramine HCl; (8) phenyl-benzyl-aminomethyl-imidazoline sulphate. The tested antihistamines prolonged clotting time.

TABLE I

EFFECTS OF REAGENTS ON CLOTTING TIME

Reagent	$d \log tc/dc$(g/liter)	Pharmacological properties
Compounds known as antihistamines		
Prophenpyridamine maleate	0.271	Antihistamine
Methyl-amino-N-thenyl-piperidin tartrate	0.240	Antihistamine
Chlorprophenpyridamine maleate	0.180	Antihistamine
Mepyramine maleate	0.180	Antihistamine
Diphenhydramine HCl	0.070	Antihistamine
Phenyl-benzyl-aminomethyl-imidazaline sulphate	0.060	Antihistamine
Tranquilizers derived from phenothiazine		
Chlorpromazine HCl	Precipitates fibrinogen	Tranquilizer, Antihistamine, Hypnotic
Promazine HCl	Precipitates fibrinogen	Tranquilizer, Antihistamine
Mepazine HCl	0.00	Tranquilizer
Diethazine HCl	0.00	Anti-parkinsonian
Promethazin HCl	0.100	Antihistamine
Compounds derived from diphenylmethan		
Azacyclanol	0.00	Tranquilizer
Benactyzine	0.00	Tranquilizer, Taming effect, Spasmolytic
Adiphenine	0.00	Spasmolytic
SKF 525-A	0.00	
Chlorcyclizine HCl	0.090	Antihistamine
Compounds derived from substituted propanediols		
Meprobamate	0.00	Muscle relaxant, Tranquilizer
Metuchen	0.00	Inactive
Mephenesin	0.00	Tranquilizer
Glycetal	0.00	Tranquilizer
Others		
Reserpin	0.00	Sedative, Taming effect
Deserpedin	0.00	Liberate Serotonin in the brain

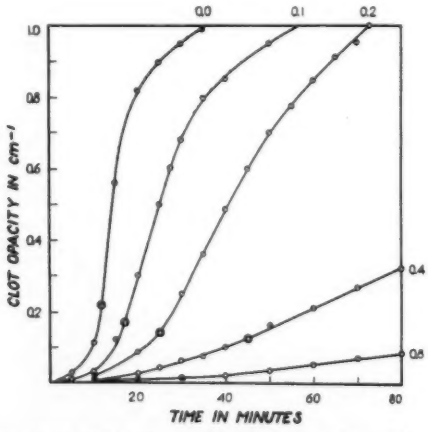

FIG. 2. Clot opacities plotted against time in minutes for different concentrations of an antihistamine (mepyramine maleate). The antihistamine decreased clot opacity at clotting time (⊙). Concentration of fibrinogen was 5 g/liter in the system: pH 6.3, ionic strength 0.45, units/ml thrombin.

(3) Effect of Reagents on Clot Opacity

The effects of reagents on the conversion of fibrinogen to fibrin are also reflected in the clot-opacity curves. The clot opacity rises during the conversion and reaches a characteristic opacity at the time of clotting. Figure 2 shows opacity curves obtained by using different amounts of mepyramine maleate in the inhibited system containing 5 g/liter fibrinogen. It can be seen that this antihistamine decreases the rate of fibrin formation, and clot opacity at time of clotting decreases constantly with mounting doses of the antihistamine. All the other antihistamines tested similarly reduced the clot opacity at time of clotting.

(4) Reversibility of Interaction between the Antihistamines and Thrombin or Fibrinogen

The following experiments suggest that the antihistamines do not cause irreversible changes in fibrinogen or thrombin.

(*a*) Each antihistamine was added separately to fibrinogen solutions under the standard experimental conditions (fibrinogen 2.5 g/liter, ionic strength 0.45, pH 6.3). Concentration of reagents was somewhat above minimum inhibiting levels. Mixtures were kept at room temperatures for 2 hours and then dialyzed against large volumes of sodium chloride – phosphate buffer at ionic strength 0.45 for 24 hours, the buffered solution being changed 4 times. No precipitation was observed. Addition of 1 unit/ml thrombin resulted in normal clotting time and normal opacity.

(*b*) The same experiments were performed by dialyzing, against sodium chloride, solutions of ionic strength 0.45. Addition of thrombin resulted in no clotting. After the pH was readjusted to 6.3, normal clotting time and opacity were obtained.

(*c*) Solutions of thrombin (25 units/ml) were allowed to stand overnight in the presence of the antihistamines. The concentration of reagents was slightly higher than the minimum inhibiting level. The next day the solutions were diluted to a concentration of 1 unit/ml of thrombin and then added to standard fibrinogen solutions in the standard conditions. Clots of normal opacity and clotting time were registered. The thrombin was not affected by the reagents.

(*d*) Mixtures containing fibrinogen, thrombin, and the reagents were prepared and allowed to stand for 5 days, whereupon they were dialyzed as above. This procedure resulted in clotting as soon as the concentration of the reagents fell below the minimum inhibiting level.

Discussion

The conversion of fibrinogen to fibrin is a polymerization brought about by end-to-end and side-by-side associations of the fibrinogen molecule. The physical properties of the polymers depend on the number of monomers participating in the association and the type of association. The characteristics of the gel formed during polymerization by the action of thrombin

were carefully studied by Ferry and co-workers (11,12,13). If polymerization occurs at a high pH or ionic strength, gelation results in a "fine clot" with low opacity, high rigidity, and slight tendency to syneresis. On lowering of the values of the pH or ionic strength, a rather "coarse clot" occurs with high opacity, low rigidity, and enhanced tendency to syneresis. Similar variations were observed by Ferry and Shulman (11) at constant ionic strength and pH values with a number of hydroxyl compounds as a result of interference with van der Waals' forces and "consequent steric interference with both end-to-end and side-by-side union of fibrinogen molecules".

We found that recalcification time, prothrombin time, prothrombin activity, and antithromboplastic activity of the rabbit blood-plasma were not influenced by any of the reagents tested. These observations supply further experimental evidence that antihistamines do not interfere with the enzymes tested in the clotting system and that the described in vitro action of the reagents is a direct interference with the polymerization of the fibrinogen. While clotting time of fibrinogen solutions was considerably altered, the clotting time of the whole rabbit blood was but slightly increased by the antihistamines as was also found by Haley and Stolarsky (14), Zetler (15), and Emmenegger and Luscher (16). It seems that the action of the reagents on clotting time of fibrinogen is less specific, and of less biological significance than their effect on opacity. The formation of characteristic intermediate polymers in the presence of the antihistamines is more specific and this action must be emphasized rather than the effect of prolonging clotting time.

Since the antihistamines, apart from prolonging clotting time, also decrease the clot opacity at the time of clotting, it seems that the compounds interact with fibrinogen rather than with thrombin. The decreased clot opacity, resulting in a "fine clot", is characteristic for gels of predominantly end-to-end associations (12, 13). The low clot opacity measured on such inhibited systems suggests that antihistamines interfere with the side-by-side association of the fibrinogen molecules, thus resulting in a fine clot. None of the reagents produced an irreversible action on fibrinogen. The effects are therefore assumed to be the results of interference with van der Waals' forces, which thus prevents the fibrinogen or profibrin molecules from approaching closely. However, it was not evident whether the reagents prevent the interaction between the fibrinogen monomer and thrombin, or whether they prevent the association of fibrinogen molecules or some intermediate proteins. Whatever the case may be, the arrest of polymerization at any instant results in a change of clotting time and clot opacity.

The question may also arise whether the observed effects on clotting time and clot opacity are the results of interaction with the maleate, hydrochloride, sulphate, or phosphate groups of the reagents. If these groups were responsible for the effects, all maleates, for instance, would have the same effect on the slope of the clotting time as expressed by $d\log tc/dc$. However, this is not the case since the various maleates gave very different slopes (0.180–0.271), although having nearly the same molecular weight. The same can be said for antihistamines and amino acids and their hydrochlorides;

for instance Tryptamine HCl and *d*-arginine HCl had no effect on $d\log tc/dc$. Furthermore, the dissociability of the compounds is extremely low in the phosphate-buffer solution used. The effects cannot be considered either as results of changes in the ionic strength or pH, since these were practically not altered by the moderate amounts of reagents present in the system. The observed effects therefore must be looked for in the specific structural configurations or energy characteristics of the molecules of the reagents.

The ultimate objective of the present investigation is to elucidate in terms of biochemical reactions some of the phenomena connected with the defence process of the living organism against injury. Taking into consideration our observations that antihistamines inhibit the phagocytic activity of fixed and mobile cells of the RES even in the absence of extrinsic histamine in vitro (2,3) and in histamine depleted rats (4,5), we cannot exclude the possibility that direct effect of antihistamines on the specific cells might be at least in part the result of surface effect by inducing characteristic modifications in the geometry of polymerization as evidenced by the changes in clot opacity. Interaction of antihistamines and fibrinogen resulted in a decreased clot opacity in the presence of thrombin, thus resembling the "fine clot" with high rigidity and slight tendency to syneresis. If by means of appropriate methodology adequate data could be obtained for a similar effect of the antihistamines in vivo, the direct effect of these compounds on the RES cells could be explained as a specific change in the physical properties of the environment in which phagocytic cells or the capillary endothelium perform their physiological functions. We must, however, fully recognize that the present observations have but limited and theoretical bearings. The fact that the inhibiting effect on fibrinogen polymerization among the compounds tested is specific for those having antihistaminic properties and that this effect is reversible, underlines the intriguing conclusions of Macfarlane (17) that "the mechanism of coagulation is only a part of a far more complex mechanism by which the blood reacts to injury" and that "coagulation may have wider ramifications than its obvious hemostatic effect".

References

1. Gözsy, B. and Kátó, L. Can. J. Microbiol. **1**, 461 (1955).
2. Kátó, L. and Gözsy, B. Can. J. Biochem. and Physiol. **34**, 580 (1956).
3. Kátó, L. and Gözsy, B. Internat. J. Leprosy, **24**, 447 (1957).
4. Gözsy, B. and Kátó, L. Arch. intern. pharmacodynamie, **112**, 235 (1957).
5. Gözsy, B. and Kátó, L. J. Physiol. **139**, 1 (1957).
6. Gözsy, B. and Kátó, L. Studies on phagocytic stimulation. Monograph. Institute of Microbiology and Hygiene, University of Montreal, Canada. 1957.
7. Biggs, R. and Macfarlane, R. G. Human blood coagulation. Charles C. Thomas, Publisher, Springfield, Ill. 1953.
8. Montigel, C. and Pulver, R. Schweiz. med. Wochschr. **82**, 132 (1952).
9. Tocantins, L. M. Am. J. Physiol. **139**, 265 (1943).
10. Morrison, P. R. J. Am. Chem. Soc. **69**, 2723 (1947).
11. Ferry, J. D. and Shulman, S. J. Am. Chem. Soc. **71**, 3198 (1949).
12. Ferry, J. D. and Morrison, P. R. J. Am. Chem. Soc. **69**, 388 (1947).
13. Ferry, J. D. Advances in protein chemistry, **4**, 2 (1948).
14. Zetler, G. Arch. exptl. Pathol. Pharmakol., Naunyn-Schmiedeberg's, **213**, 18 (1951).
15. Haley, Th. and Stolarsky, F. Proc. Soc. Exptl. Biol. Med. **76**, 299 (1951).
16. Emmenegger, H. and Luscher, E. Helv. Physiol. et Pharmacol. Acta, 12, C-13 (1954).
17. Macfarlane, R. G. Physiol. Revs. **36**, 479 (1956).

PAROTID SECRETION OF PROTEIN IN MAN[1]

MARION H. FERGUSON, H. P. KRAHN, AND J. A. HILDES

Abstract

In unstimulated saliva, total protein concentration averaged 186 mg per 100 ml and amylase activity 146 units per 100 ml. The protein concentration was lower in the early morning than at midday. After dilute acetic acid stimulation, both total protein concentration and amylase activity were increased but the concentrations were not affected by rates of secretion above 0.1 ml per minute. Unlike protein, the potassium concentration fell with stimulation.

Using zone electrophoresis on filter paper, as many as nine protein components were found, none of which corresponded to the serum proteins. The amylase activity was restricted to a component of low mobility which moved to the anode. There were two or three bands containing glycoproteins; all moved towards the cathode. There were qualitative and quantitative differences between stimulated and unstimulated secretions. Saliva collected 2 or 24 hours after a tracer dose of I^{131} showed less than 1% residual radioactivity after dialysis or treatment with an anion exchange resin, indicating that little if any of the salivary iodide is organically bound.

Introduction

It has been reported that in the submaxillary saliva of dogs and cats there is a direct relationship between the concentration of organic constituents and the rate of secretion (1, 2, 3). From studies using only two rates of secretion, "fast" and "slow", Bramkamp (4) and Araki (5) have concluded that this same phenomenon occurs in human parotid saliva. On the other hand, Faber (6) reported that in normal humans the protein content of mixed saliva was inversely related to the rate of secretion. Schneyer (7) found the amylase content of human parotid juice to be independent of a moderate increase in rate.

Other factors have also been said to influence the concentration of the organic constituents. Baxter reported that in a dog with a parotid fistula different oral stimuli produced secretions of differing protein content at similar flow rates (8); and in humans Squires (9) found variations in amylase activity of whole saliva depending on the carbohydrate content of the diet. Walker and Sheppard (10) showed that there is a marked variation in amylase activity of whole saliva in the same individual at different times of the day, the activity being highest after meals and least after a night's fast.

With regard to the type of protein in saliva, the presence of amylase is well known. There have been recent attempts to characterize the other proteins further. Bramkamp (4) reported that the protein of parotid saliva was principally albumin, as determined by salting out procedures. Although studies of the protein of mixed saliva by Kinersly (11, 12), using zone electrophoresis on filter paper, showed four fractions, and studies by Zipkin *et al.* (13),

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using boundary electrophoresis, showed seven distinct peaks in parotid juice, none of these fractions had the mobility of serum albumin. Weiss and Burgen (14) have reported that the iodide in dogs' parotid juice is 10–40% protein bound. However, this has not been found in humans (15, 16).

The present report deals with studies on human parotid juice in which the relationship of total protein concentration and of amylase activity to the rate of salivary secretion was observed. The electrophoretic pattern of both stimulated and "resting" saliva and observations on the nature of iodide in saliva are also presented.

Methods

The subjects were healthy adults of the staff and student body. Except as noted below, the subjects attended the laboratory in the morning without breakfast. Parotid juice from each gland was collected in graduated tubes, using double-walled cups held over the papillae by suction as previously described (17). The flow rate was determined by timing the collections. At least 1 ml of saliva was collected; at very low rates this required up to an hour; at fast rates collections were made for at least 1 minute. To study very low rates of secretion, unstimulated or resting saliva was collected while faster rates were produced by gustatory stimulation with dilute acetic acid.

In the iodide experiments parotid juice was collected as described and mixed submaxillary and sublingual saliva was collected using a dental suction. Collections were made after intervals of either 2 or 24 hours after the ingestion of a tracer dose of 30–60 μc of I^{131}. The radioactivity of the saliva was measured (18) both before and after either dialysis in cellophane bags against tap water or passage through an anion exchange resin column (19).

Analytical Methods

Total protein was determined by the biuret method (20). The coefficient of variation for this method, based on 20 duplicates, was found to be 1.0%.

Amylase was measured by a modification of the method of Smith and Roe (21), in which the starch substrate was 2.4% instead of 1.2%. The method is based on the photometric measurement of the blue color of the residual iodine–starch complex after incubation, using a standard amount of fresh starch as substrate. The color was read immediately in a Beckman DU spectrophotometer at 610 mμ. The results were expressed in units of amylase activity per 100 ml saliva. For blood, Smith and Roe defined one unit of amylase activity as being the amount of amylase which in their method, using 60 mg of starch as substrate, will hydrolyze 10 mg of starch in 30 minutes. Our amylase unit for saliva is defined as the amount of amylase which will hydrolyze 10 g of starch in 30 minutes. For this measurement the saliva was diluted 1000 or 2000 times.

To test the precision of this method, 10–16 determinations were carried out on each of five specimens of saliva. The coefficient of variation varied from 4% to 5%.

Potassium concentration was estimated using a Barclay flame photometer with a barrier layer type photocell and an internal standard.

Electrophoresis was carried out on the pooled saliva from both parotid glands. Total protein concentration and amylase activity were determined and 5 ml of the pooled saliva was placed in a cellulose bag and dialyzed against tap water at 10° C for 48 hours. The contents of the bag were then dried over phosphorus pentoxide in a vacuum desiccator and reconstituted in 0.2 ml of barbitone buffer, pH 8.6, and ionic strength 0.05; 0.02 ml of the solution was then applied to each of two to four filter paper strips which were mounted in a Spinco Model R electrophoresis apparatus and electrophoresed simultaneously for approximately 12 hours with a current of 1.0 ma per cm width of filter paper.

Some of the strips were oven-dried, stained with bromphenol blue (22), and scanned (Spinco Analytrol). Other strips were cut into sections 0.5 cm. wide and eluted with the starch – buffer – sodium chloride solution used in the amylase determinations, and the eluents were analyzed for amylase activity. Some strips were stained for protein-bound polysaccharides using the periodic acid – Schiff stain (23).

Results

Salivary Protein and Secretion Rate

The relationship between the secretion rate and the concentration of protein and of amylase was examined in 10 experiments on seven subjects. In these experiments approximately 1 milliliter of saliva was discarded at the beginning of each period of collection in order to wash out the dead space of the collecting system and, as far as possible, to ensure a steady rate of flow throughout the period. In these experiments no effort was made to achieve minimum flow rates, the low rates being attained by withholding the acetic acid stimulus. An experiment of this kind lasted 3 to 5 hours and permitted 10 to 40 collections taken in random order so far as rate of secretion was concerned.

The result of a typical experiment, shown in Fig. 1, indicates that there was no relationship between rate of secretion and the concentration of protein or

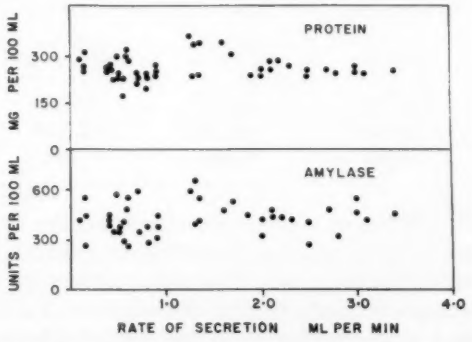

FIG. 1. Relationship between rates of secretion greater than 0.1 ml/min and protein and amylase concentration in one subject.

TABLE I

PAROTID PROTEIN AND AMYLASE CONCENTRATION AT FOUR SECRETION RATES
(Means of average values in each experiment (seven subjects))

Rate of secretion, ml/min	Number of experiments	Proteins, mg/100 ml	Amylase, units/100 ml.
<0.5	10	305±120* (10)†	607±432 (11)
0.5–1.0	10	259±68 (7)	454±179 (7)
1.0–2.0	10	280±78 (8)	550±316 (9)
>2.0	8	273±94 (6)	551±234 (6)

*Standard deviation.
†Average number of observations per experiment.

the amylase activity. This is confirmed in Table I which presents a summary of all the results in this series of experiments.

The coefficient of correlation between the total protein concentration and amylase activity for each experiment was highly significant, the P value in each instance being less than .01.

The results appeared to contradict the conclusion (4, 5) that the concentration of the organic constituents varies with the rate of flow. Therefore, another set of experiments was undertaken to explore the relationship at very low rates of flow prior to stimulation. Six experiments were performed on four experienced subjects. Care was taken in applying the collecting cups to minimize stimulation of the mucosa so that a sample at a low flow rate was obtained for comparison with one obtained subsequent to acetic acid stimulation. In each experiment samples were collected from right and left glands, thus providing 12 pairs for comparison. The results are shown in Table II.

TABLE II

THE EFFECT OF STIMULATION ON SALIVARY RATE AND COMPOSITION

	Rate, ml/min	Protein, mg/100 ml	Amylase, units/100 ml	Potassium, meq/l
Resting	0.05±0.01*	186±16	146±20	28.8±3.4
Stimulated	1.26±0.17	419±34	661±79	17.1±0.5
P†	<.001	<.001	<.001	<.001

*Standard error of the mean.
†Based on analysis of paired data.

It will be seen that with acetic acid stimulation the mean rate of secretion changed from 0.05 to 1.3 ml/min. The mean percentage increase in the protein concentration was 139% and in the amylase concentration 447%, while the potassium concentration decreased 34%. The interpretation of these results is discussed below.

In Table I it will be seen that the standard deviations of the protein and amylase concentrations were relatively large, indicating that there was a considerable variation from experiment to experiment. In an attempt to identify some of the factors responsible for the variation, the following experiments were performed. Repeated stimulation to produce high flow rates for as long as 5 hours had no effect on protein and amylase concentrations. Sucrose stimulation gave results similar to acetic acid stimulation. The protein concentration at various rates of flow from 0.1 to 2 ml/min was measured at 6.30 a.m. as soon as the subject wakened and compared with that in the midmorning after breakfast (Table III). In 9 of 10 experiments on six subjects the average protein concentration was lower in the early morning specimens, the mean of all experiments being 156 mg/100 ml for the early morning and 259 mg/100 ml for the late morning observations. The serum protein was measured in six of these experiments and in all instances was greater in the second set of measurements, the average for the early morning values being 6.96 g/100 ml and for the late morning 7.38 g/100 ml. These results indicate a consistent diurnal variation but do not permit identification of the responsible factor. This set of experiments, incidentally, confirmed the results in Table I which showed that, at rates of flow over 0.1 ml/min, the protein concentration was not related to the flow rate.

TABLE III

SALIVARY AND PLASMA PROTEIN IN EARLY MORNING AND AT NOON
(Secretion rate 0.1–2.0 ml/min)

Subject	Mean parotid protein, mg/100 ml		Plasma protein, g/100 ml	
	6.30 a.m.	12.00 noon	6.30 a.m.	12.00 noon
1*a*	75	147		
b	102	198		
2*a*	201	360		
b	264	321		
3*a*	154	242	7.10	7.41
b	179	288	7.27	7.51
4*a*	289	187	7.17	7.44
b	143	254	7.17	7.30
5	123	279	6.62	7.54
6	143	179	6.45	7.06

Electrophoretic Pattern

Examples of the electrophoretic patterns are shown in Fig. 2. On examination of the stained strips nine different bands may be identified. The proteins forming bands 1–3 moved to the anode and bands 4–9 to the cathode. The frequency with which the various bands were seen is shown in Table IV. Band 3 was always present, bands 5 and 6 almost always, and the remaining less frequently. More bands were usually seen in the stimulated samples. Band

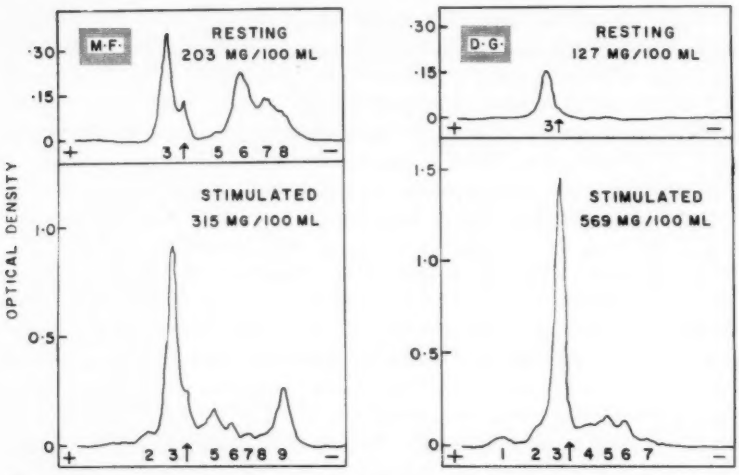

FIG. 2. Electrophoretic pattern of resting and stimulated parotid saliva from two subjects.

TABLE IV

ELECTROPHORETIC BANDS IN PAROTID SALIVA

	Incidence in five tests on different subjects		Incidence in seven tests on one subject	
Bands	Resting	Stimulated	Resting	Stimulated
1	1/5	4/5	0/7	2/7
2	2/5	5/5	1/7	7/7
3	5/5	5/5	7/7	7/7
4	2/5	1/5	1/7	1/7
5	4/5	5/5	7/7	7/7
6	4/5	5/5	7/7	7/7
7	2/5	4/5	5/7	5/7
8	2/5	4/5	6/7	5/7
9	0/5	4/5	3/7	7/7

3 was usually the most deeply stained and, although this procedure is considered to be only very roughly quantitative, it seems that protein of this mobility formed the bulk of the salivary protein. The differences between resting and stimulated saliva and between subjects are also shown in Fig. 2. Variations in the same subject tested on different occasions were also noted; an example of this is shown in Table IV. These results indicate that there were qualitative as well as quantitative differences between resting and stimulated saliva. This is also indicated by the data presented in Table II which show that the ratio between protein and amylase concentration was very different under resting and stimulated conditions.

In three experiments comparison of the electrophoretic pattern of parotid saliva and simultaneously collected mixed submaxillary and sublingual saliva

from the floor of the mouth showed that the latter had an entirely different pattern with one main band moving to the cathode with the mobility of band 3.

In order to determine the nature of the proteins in the different bands, four methods have been used.

Blood serum was diluted approximately 20 times to a protein concentration similar to that found in parotid saliva. It was then dialyzed, dehydrated, reconstituted, and analyzed electrophoretically as described above for salivary proteins. This treatment did not change the electrophoretic pattern of the serum proteins. Such diluted serum and parotid juice from the same subject were analyzed both separately and after being mixed in equal proportions. The components of each were completely separated electrophoretically, suggesting that saliva and serum have no common protein components.

Fifteen strips were cut up, eluted, and tested for amylase activity; all showed that the amylase activity is confined to band 3.

The electrophoretic strips from three subjects, which were stained with periodic acid – Schiff stain showed staining in bands 5, 6, and 7 and possibly 4, indicating the presence of polysaccharides in these bands.

Two normal healthy subjects were given a tracer dose of 40 and 60 μc I^{131} respectively by mouth and parotid saliva was collected 2 hours later. The saliva contained the usual high concentration of I^{131} (18) but after 48 hours' dialysis no radioactivity remained. In one of these subjects mixed saliva from the other glands was also collected with similar results. As the 2-hour period might be considered too short for protein binding of the iodide, this experiment was repeated on three euthyroid hospital patients. In this case total mixed saliva was collected 24 hours after the oral administration of a tracer dose of I^{131}. Again the initial high salivary radioactivity was removed by dialysis. In two of these samples a precipitate formed in the saliva which still retained 1% of the initial radioactivity after 60 hours' dialysis. In another three subjects, saliva collected 24 hours after a tracer dose of I^{131} was treated by passage through an anion exchange column to remove inorganic iodide. There was no residual radioactivity detectable after this treatment, indicating that human saliva contains no iodide-binding protein.

Discussion

The present results, which show no relationship between protein or amylase concentration in parotid saliva and the rate of secretion at rates above 0.1 ml/min, are contrary to reported results in cats and dogs (1, 2, 3) and to some reports in humans (4, 5, 6, 7). The change in resting salivary composition caused by stimulation (Table II) may be due to the stimulation or may be due to a change in the composition at low rates of secretion. The difference in the electrophoretic pattern between resting and stimulated saliva tends to support the former view, in that stimulation affects not only the rate but the composition as well.

The opposite relationship at very low rates of secretion between rate of secretion and protein concentration on the one hand and potassium concentration on the other does not support the hypothesis that water is reabsorbed from the duct at very low rates of secretion (24). Burgen (25) has shown that this simple explanation for the high potassium content of saliva at low secretion rates is untenable and has proposed a theory of salivary secretion which includes the reabsorption of potassium from the ducts. However, the qualitative differences in protein composition of the saliva at low and high rates of secretion suggest that differences in secretory activity rather than in reabsorption may be responsible for the observed variations in potassium concentration.

The significant correlation between protein and amylase in stimulated saliva suggests either that the amylase forms a high proportion of the total protein or it forms a constant proportion of the total protein. The localization of amylase on electrophoresis to the most heavily stained band lends some support to the former hypothesis.

The amylase moves consistently at a relatively slow rate towards the anode. Glycoproteins identified by the PAS reaction move towards the cathode. The results to date indicate that there is probably more than one glycoprotein in saliva. The remaining protein bands have not yet been identified; they do not seem to be identical with any of the blood proteins nor does it seem that any of them contain protein-bound iodine.

Acknowledgment

We are grateful to Miss K. L. Rasmussen for technical assistance.

References

1. Heidenhain, R. Pflüger's Arch. ges. Physiol. **17**, 1 (1878).
2. Langley, J. N. and Fletcher, H. M. Phil. Trans. Roy. Soc. London. Ser. B, **180**, 109 (1889).
3. Langstroth, G. O., McRae, D. R., and Stavraky, G. W. Proc. Roy. Soc. (London), B, **125**, 335 (1938).
4. Bramkamp, R. G. J. Biol. Chem. **114**, 369 (1936).
5. Araki, Y. Japan. J. Physiol. **2**, 69 (1951).
6. Faber, M. Acta Med. Scand. **118**, 457 (1944).
7. Schneyer, L. H. J. Appl. Physiol. **9**, 453 (1956).
8. Baxter, H. Am. J. Physiol. **91**, 132 (1930).
9. Squires, B. T. J. Physiol. **119**, 153 (1953).
10. Walker, F. and Sheppard, L. Am. J. Physiol. **111**, 192 (1935).
11. Kinersly, T. Yale J. Biol. and Med. **26**, 211 (1953).
12. Kinersly, T. and Leite, H. B. Yale J. Biol. and Med. **29**, 496 (1957).
13. Zipkin, I., Adamik, E. R., and Saroff, H. A. Proc. Soc. Exptl. Biol. Med. **95**, 69 (1957).
14. Weiss, M. and Burgen, A. S. V. Proc. Can. Physiol. Soc. Ottawa, 66 (1957).
15. Honour, A. J., Myant, N. B., and Rowlands, E. N. Clin. Sci. **11**, 447 (1952).
16. Freinkel, N. and Ingbar, S. H. New Engl. J. Med. **252**, 125 (1955).
17. Hildes, J. A. and Ferguson, M. H. Can. J. Biochem. and Physiol. **33**, 217 (1955).
18. Ferguson, M. H., Naimark, A., and Hildes, J. A. Can. J. Biochem. and Physiol. **34**, 683 (1956).
19. Slade, C. I. J. Clin. Endocrinol. and Metabolism, **16**, 1122 (1956).
20. Gornall, A. G., Bardawill, C. J., and David, M. M. J. Biol. Chem. **177**, 751 (1946).
21. Smith, B. W. and Roe, J. H. J. Biol. Chem. **179**, 53 (1949).
22. Durrum, E. L. J. Am. Chem. Soc. **72**, 2943 (1950).
23. Bjornesjo, K. B. Scand. J. Clin. & Lab. Invest. **7**, 153 (1955).
24. Babkin, B. P. Secretory mechanisms of the digestive glands. 2nd ed. Paul B. Hoeber, Inc., New York. 1950.
25. Burgen, A. S. V. J. Physiol. **132**, 20 (1956).

PROBLEMS ASSOCIATED WITH THE USE OF THE EXPOSED PLATINUM ELECTRODE FOR MEASURING OXYGEN TENSION IN VIVO[1]

WILLIAM RODGER INCH[2]

Abstract

The equation governing the distribution of oxygen around a stationary exposed platinum electrode was solved for the size of electrode used and for a number of different times. In protein solutions the oxygen polarogram could be satisfactorily monitored by measuring the diffusion current at −0.6 and −0.7 volts. After using the platinum electrode in solutions containing protein it was found necessary and sufficient to clean it in acid dichromate solution followed by neutralization in a buffer solution. This procedure reproduced the original diffusion current. In solutions containing heterogeneities with small coefficients of oxygen diffusion, the electrode current was found to be less than when the heterogeneities were not present.

Introduction

The use of a small stationary exposed platinum electrode as the polarizable half of a polarographic cell has been described by several investigators (1, 2, 3, 4). In many aqueous solutions the application of a suitable voltage across such a cell results in a diffusion current which is proportional to the concentration of oxygen dissolved in the liquid phase. Brink and Davies were among the first workers to construct working electrodes and apply the technique to biological problems (4). They examined the electrode reaction and from their experimental data calculated a coefficient of oxygen diffusion in saline (3.5×10^{-5} cm^2/sec at 25° C) which agreed very well with accepted values.

Montgomery and Horvitz used a solid platinum electrode for measuring the variations of oxygen tension in human skin and found that it depended very greatly on blood flow (5). They calibrated the method by measuring diffusion currents when the electrode was inserted into freshly excised skin in equilibrium with saline at different oxygen concentrations. On the average the current was about one quarter of the value in saline but the reason for this was not discussed. In addition, they studied the effect which changes in temperature had on the electrode current and found a variation amounting to approximately 2% per ° C.

Other investigators have adopted the electrode and technique developed by the above workers and have proceeded to measure and compare diffusion currents in a number of different physiological situations, assuming that

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they were measuring oxygen tension or concentration (6, 7). It is unfortunate that more of this effort was not devoted to the analysis of some of the effects which are readily demonstrable in vitro and which obviously operate when the exposed electrode is used in tissues. It was felt that even the calibration of the exposed platinum electrode in plasma, blood, skin, and glycerin might assist in elucidating a few of these problems.

At the same time it was realized that in vivo the technique has another complication. In addition to the mere exposure of the platinum surface to protein, the presence of physical barriers to diffusion in the form of cell membranes, etc., significantly alters the oxygen distribution around the electrode tip during electrolysis. This, in turn, leads to variations in the electrode current which are independent of the oxygen concentration.

Method

In the design of a polarizable electrode for measuring the concentration of oxygen within a volume of tissue it was considered essential that at least the following two conditions be satisfied:

(1) The physical size should be as small as possible. This is necessary not only because it facilitates insertion into the tissue and results in less cellular damage, but also because the spatial resolution is correspondingly better and oxygen gradients which are known to exist in tissues (8) will have less influence on the diffusion current.

(2) The time for any one measurement should be as short as possible. This determines the resolution which the electrode will have for an oxygen field which is changing with time. In experiments where the oxygen consumption of cells is being measured by occluding the oxygen supply, this plays an extremely important role.

Several polarizable electrodes, all of the solid type, were considered: (*a*) an exposed tip, either stationary or vibrating (5, 3); (*b*) similar to (*a*) but having the tip covered with a membrane of collodion or cellophane (9); (*c*) the active surface recessed, the recess being filled with agar, or gelatin, or saline (4).

Preliminary experiments indicated that the introduction of substance (whether membrane, gel, or saline) between the active electrode surface and the solution to be measured increased the time required to reach equilibrium conditions. This increase depends on the thickness of the interposing substance and its oxygen diffusion coefficient. Although electrode poisoning in protein solutions was less in all of these cases it was not eliminated. Presumably this was due to the attractive force of the electric field which pulled protein molecules into the membrane pores or into the gel matrix; in the case of the recessed type filled with saline, protein would eventually deposit on the platinum surface.

The solid vibrating electrode offered at least two advantages over the same size stationary type: (*a*) a larger diffusion current and (*b*) a greater

spatial resolution. When used in protein solutions the diffusion current is quite stable; however, in tissues the current is largely dependent on the physical reaction of the tissue to the vibration (whether the motion is along the electrode axis or at right angles to it) and because of this it was considered unsuitable.

It was therefore decided that the most useful polarizable electrode for in vivo experiments was the stationary exposed platinum type, made as small as possible. Gold, as the metallic element, appears to have some advantage over platinum because of its higher overvoltage (10); however, the former metal proved more difficult to insulate and therefore was not used.

The actual construction was carried out in a manner similar to that described by Montgomery and Horvitz. The data to be reported were obtained using electrodes made from 0.18-mm diameter pure platinum wire (obtained from Johnson, Matthey, and Mallory, Toronto). Little difficulty was experienced in producing stable electrodes providing that they were properly annealed and care was taken during the grinding of the tip (60° right cone) so that a good platinum–glass junction was maintained. Working electrodes were chosen on the basis of the reproducibility of the oxygen diffusion current measured after 30 seconds, in aerated saline, corresponding to a potential of -0.6 volts, and standardized to a temperature of 20° C. It was found that the diffusion current was more consistent if the electrodes were stored in saline and were not allowed to dry out. Approximately one electrode in four was rejected because this diffusion current was not reproducible (i.e. in a series of readings the variation in current exceeded 5%). The exact reason for this instability is not known. During the first few days after an electrode tip has been formed, there is a slight decrease in current which is attributed to oxidation of the platinum surface (10). Also, in a series of readings in which the circuit is made and broken a number of times one must take into account the time required for polarization of the electrode; assuming that complete polarization does not occur in a single reading, then comparable currents will be obtained only if the time intervals are kept the same.

The external circuit was completed through an unsaturated calomel half cell, having an aqueous phase of 0.9% KCl (mercurous chloride and electrolytic mercury obtained from Eppley Laboratory Inc., Newport).

The remainder of the circuit consisted of a modified version of the model XII Sargeant–Heyrovsky polarograph (O. H. Sargeant & Company, Chicago). This instrument had a current sensitivity of 0.7×10^{-8} amp/mm, a period of 4.4 seconds, and a photographic recorder which could be used to give either a current–voltage or current–time record.

Theoretical Analysis of Electrode Reaction on Closing Circuit

After the circuit has been closed, the distribution of oxygen around the electrode tip is governed very closely by the laws of symmetrical spherical diffusion.

The fundamental differential equation describing symmetrical spherical diffusion up to a stationary spherical electrode is given by (6):

$$[1] \qquad \delta C/\delta t = D\,[\delta^2 C/\delta r^2 + 2/r(\delta C/\delta r)]$$

which has a particular solution.

$$[2] \qquad C_{r,t} = C\left(1 - \frac{r_0}{r}\right) + \frac{2Cr_0}{\sqrt{\pi}r}\int_0^{\frac{r-r_0}{2\sqrt{(Dt)}}} e^{-y^2}dy.$$

Using equation [2] it can be calculated that the instantaneous current is given by,

$$[3] \qquad i_t = nFADC\ (1/r_0 + 1/\sqrt{(\pi Dt)}).$$

In these equations C represents the concentration of oxygen; D, the coefficient of oxygen diffusion; r is the distance measured from the center of the sphere, i.e. electrode, whose radius is r_0; y is a variable of integration; F is the Faraday; n is the number of electrons involved in the electrode reaction to reduce 1 mole of oxygen; and A is the active area of the electrode tip.

From equation [3] it is observed that the current approaches a constant value with time which depends primarily on the active area and to a lesser

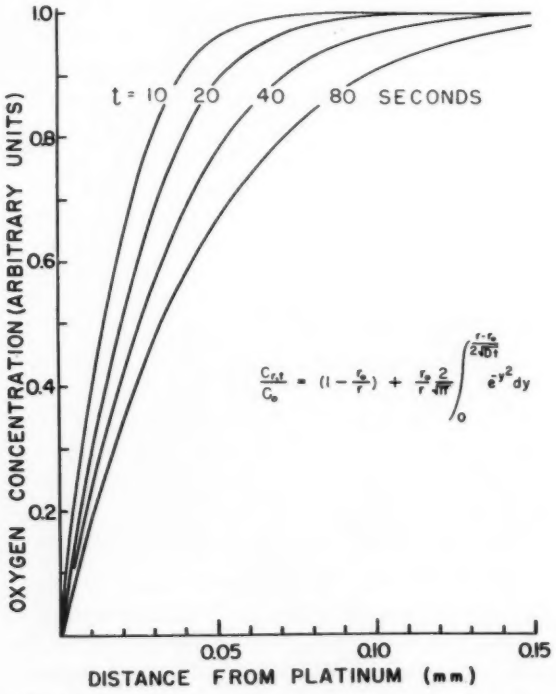

FIG. 1. Distribution of oxygen around electrode tip for various times of current flow.

extent on the physical shape of the electrode. Equation [2] has been plotted in Fig. 1 for several values of t; it is observed that the curve approaches a hyperbola with increasing time. These curves differ in shape only slightly from those applying to the linear diffusion recessed electrode (4). It is at once apparent that at the cellular level the electrode draws oxygen from a large volume around its active tip, a volume measured in terms of many cell diameters.

Results

When the polarographic technique is used, the first thing which must be investigated is the shape of the current–voltage curve (polarogram) in the solution being tested.

The normal curve for dissolved oxygen has been described by other workers (4, 11). Its fundamental characteristic is a "plateau region" between −0.3 and −0.9 volts which consists of two waves due to the two-step reduction of oxygen with the intermediate formation of hydrogen peroxide. In solid electrode polarography these two waves are not distinguishable and the width and shape of the plateau depend to a large extent on the electrolytes in the solution being measured, and to a lesser extent on the steady state conditions around the electrode tip.

In most experiments the procedure used by the author was to measure the current for 30 seconds, at a particular voltage, and then to re-equilibrate

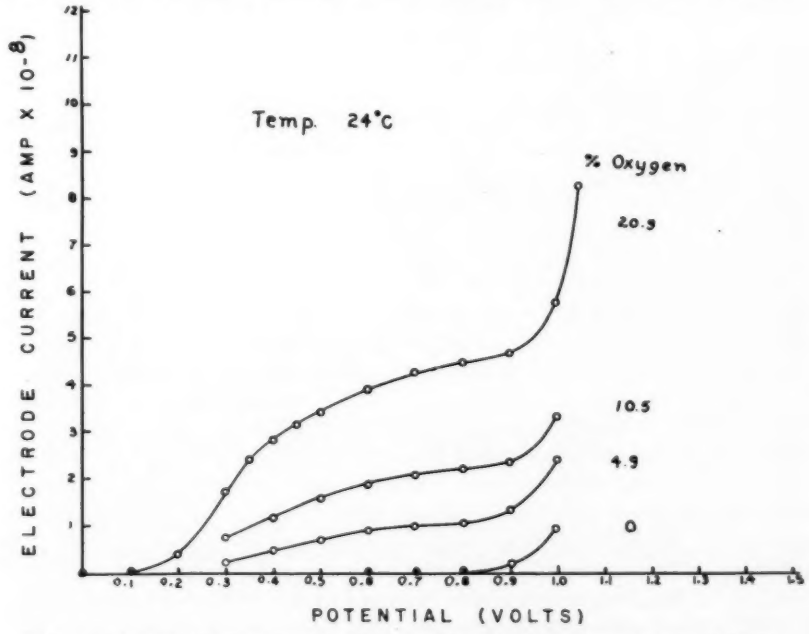

FIG. 2. Current–voltage curves in saline equilibrated with oxygen–nitrogen mixtures.

with the gas mixture being used. Since movements in the solution could not be tolerated, this meant that readings could only be taken every 2 to 3 minutes. A series of polarograms obtained in saline solutions (24° C) at different oxygen tensions is shown in Fig. 2. In experiments where the oxygen tension was changing with time the current was left on continuously and hence two calibration curves were necessary in order to compensate for the different degrees of polarization.

When the electrode is used in protein solutions, the 'plateau region' of the current–voltage relation differs considerably from theoretical values. Although there remains a definite inflection point at approximately −0.65 volts, the plateau becomes much steeper than in saline. However, a good linear relation between the oxygen tension and diffusion current still exists. Because the reaction does not follow the laws of spherical diffusion, errors in estimating oxygen tension will be introduced when diffusion current readings in different solutions are compared. In order to follow such variations it was decided that the slope of the plateau should be measured during the experiment. This was accomplished by modifying the electrical circuit of the polarograph and introducing a timing mechanism which increased the electrolyzing voltage from −0.6 to −0.7 volts for one half of the 30-second

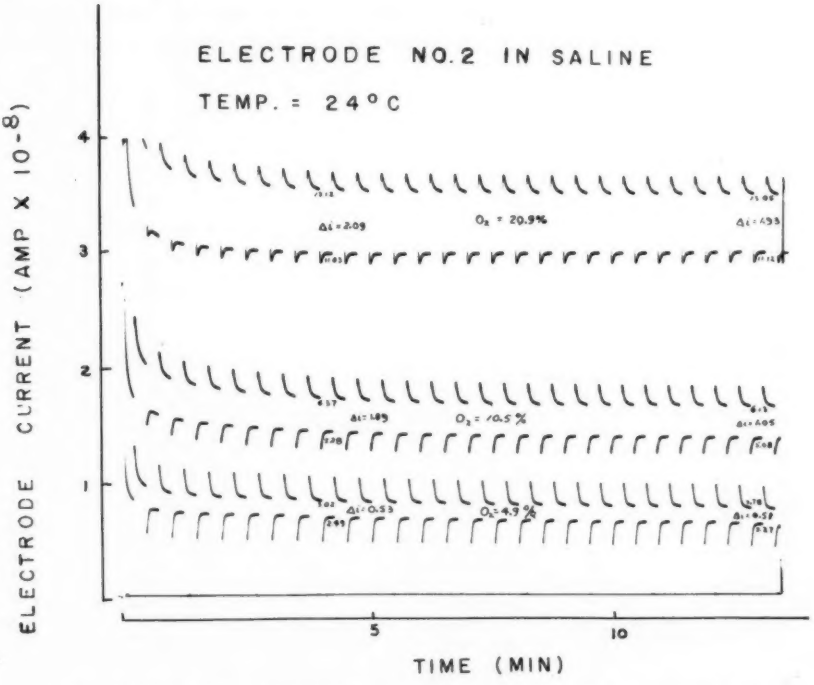

FIG. 3. Variation of electrode current with time and oxygen concentration.

period that the current was flowing. The resulting rise in current (Δi) over the voltage change (Δv) gave an index which could be used to continuously monitor the plateau. The results of this procedure are shown in an actual photographic record from the polarograph, Fig. 3. In this experiment the electrode was in saline equilibrated with various oxygen–nitrogen mixtures at a temperature of 24° C; the current was left on continuously for 14 minutes and the voltage was varied from −0.6 to −0.7 volts every 15 seconds. The decrease in the current during the first 1.5 minutes is expected from equation [3]. The ratio ($\Delta i/\Delta v$) depends on the nature and oxygen concentration of the solution, which is electrolyzed, being small in aerated saline (1.7×10^{-10} amp/volt) and up to twice this value in blood and tissue.

It is fairly certain that the current of an exposed type electrode is not diffusion-limited as is the case with the recessed type (under properly controlled conditions), but is determined by the availability of one of the substances taking part in the reaction at the platinum surface (10). From the results of previous workers (4, 11) the reduction of oxygen at the electrode tip involves at least O_2, H^+, H_2O_2, and electrons, any one of which may limit the current. It is observed from Fig. 3 that the plateau slope ($\Delta i/\Delta v$) is linearly and directly related to the oxygen concentration of the saline solution; this is also the case for plasma and blood. Whichever reactant controls the current, in nearly all biological solutions one is able to obtain a usable straight-line relation between current and tension over a wide range of oxygen concentrations, and for a number of applied voltages. Such an empirical method is quite acceptable for many problems.

Using the technique described above, diffusion currents were measured in physiological saline, plasma, whole blood, and skin, equilibrated with various oxygen–nitrogen mixtures. These are plotted in Fig. 4. When only liquids were involved the solutions were contained in a perspex chamber (volume 3.1 cc) which was supported by a small-bore glass U-tube connected to the unsaturated calomel cell. Four holes (3-mm diameter) were drilled through the cover of this chamber, through which the platinum electrode, a thermocouple, a bubbler, and a gas outlet could be introduced; rubber tubing formed gas-tight seals. A magnetic stirrer was also provided to insure complete mixing of the solution. In order to observe the effect of temperature on the diffusion current the chamber could be lowered into a temperature-controlled thermos flask. It was found that in the range 4° C to 36° C, a constant correction of approximately 1.8%/° C had to be applied to the readings. In addition it was observed that with the test chamber in the thermos flask, fluctuations in diffusion current resulting from thermal agitation were eliminated and this procedure was adopted for routine calibration.

Bubbling gases through liquids containing protein invariably produces considerable froth. Hence these solutions were equilibrated in a larger vessel and, using a syringe (5-cc capacity with 26-gauge needle), a volume was withdrawn from the bottom of the vessel and transferred to the small plastic test chamber which had been previously flushed with the equilibrating

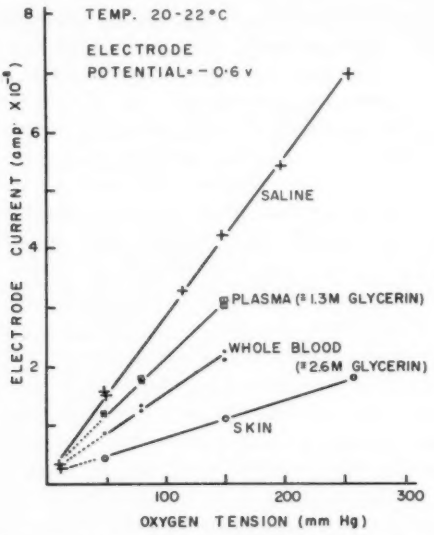

FIG. 4. Electrode current in saline, glycerin plasma, whole blood, and skin as a function of oxygen tension.

gas. Introduction was made through a rubber membrane covering one of the holes in the top of the chamber, a procedure which was found to give reproducible readings.

When the active surface of the electrode is exposed to protein solutions the diffusion current decreases at a rate of approximately 5% per hour, depending on the concentration of protein in the solution. If a d-c voltage is applied during this period the decrease is even more rapid. This effect was considered to be both a passive depositing and active electroplating of protein onto the platinum surface. No method was found for preventing this 'plating-on' so that in long-term experiments an appropriate correction factor had to be applied to all readings. This correction consisted of taking diffusion current readings in saline, at the beginning and end of the experiment, correcting these for temperature and, providing the experimental readings were taken at a constant frequency, assuming a linear relation with time.

Various methods were tried to eliminate the protein layer at the end of an experiment. Reversing the current had no effect. Electrode points were reground but this invariably changed the original calibration and often destroyed the electrode completely. The method which did produce the original diffusion current was to place the electrode tip in a cleaning solution (35 cc saturated sodium dichromate in 1 liter of concentrated sulphuric acid) for 15 minutes, neutralize by agitating in phosphate buffer (pH 7.0) for at least 15 minutes, and then wash and agitate in saline for another 15 minutes. Such a procedure returned the diffusion current to within a few per cent of its original value.

Using a method similar to that described by Montgomery and Horvitz (2), a number of diffusion currents were measured with the electrode tipped with skin. As seen from Fig. 4 the readings were about one fifth of those in saline, but results were very dependent on the time after removal of the skin from its blood supply. The electrode current varied from near zero, for freshly excised skin, up to values obtained in saline, for skin which was several days old. The readings plotted were recorded between 6 and 8 hours after normal circulation had ceased. Because of the absence of blood in the excised skin one could really not expect that this would represent conditions in vivo. However, this calibration does indicate the decrease of oxygen diffusion due to cell membranes, and the several other physical barriers to diffusion, which are present within the tissues.

In addition to the irreversible decrease in diffusion current resulting from the plating of protein onto the active platinum surface, the basic level of the current in protein solutions is always lower. This was attributed to the solution's increased viscosity. In order to investigate this effect the electrode reaction was studied in a number of different concentrations of glycerin (maintained at a constant sodium chloride concentration of 0.9%). The relation between diffusion current and concentration was observed to be sigmoid over the range 1 to 5 molar, the current decreasing with increased concentration. The sharp inflection point described by Jordan (12), using a dropping mercury electrode, was not observed although, in general, our data were in agreement with those of these authors. This discrepancy could be due to differences in the supporting electrolyte but is more probably a result of using different electrode systems. The molarities of glycerin shown on Fig. 4 were values which gave the same diffusion current as was obtained in aerated plasma and whole blood. These figures represent viscosities which are approximately one half of the accepted values for these liquids. Hence, on the basis of viscosity alone, one would predict that the diffusion current would be even lower than was obtained. The presence of small amounts of free haemoglobin in both the plasma and whole blood was originally thought to be responsible for this effect, but this theory was disproved experimentally. There are several physicochemical differences between glycerin and plasma or blood which could produce an increased current and these are presently being studied.

Another observation which is common when using open-ended electrodes in vivo is the large variation in current obtained on advancing the electrode into the tissue. It is accepted that oxygen gradients, determined by the physical distribution of capillaries and metabolizing cells, exist in tissues (8), and in part this would serve to explain such a phenomenon. However, it was felt that many of the falls in diffusion current were far too large to be explained on this basis. As pointed out above, when the distribution of oxygen around an open-ended electrode (Fig. 1) is plotted, it at once becomes apparent that the volume from which oxygen is drawn is quite large. Experiments were therefore performed in which the electrode was moved toward

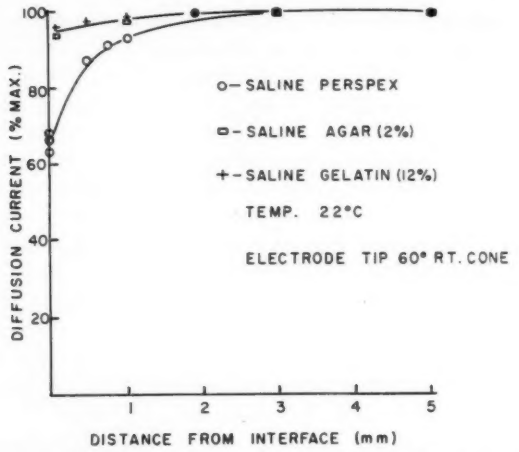

FIG. 5. Variation of electrode current with distance from plane interface, electrode tip 60° right cone.

plane interfaces of saline and perspex, saline and agar (2%), and saline and gelatin (12%). The results are plotted in Fig. 5. It is observed that as the electrode approaches the saline–perspex interface the diffusion current decreases and on contact reaches a constant value of approximately sixty-five per cent of the maximum current. This value is dependent on the physical shape of the electrode which in this case was a 60° right cone. On approaching the other interfaces, the current dropped only very slightly (gelatin 3%, agar 6%), all readings being very stable and reproducible providing the oxygen in the gelatin and agar layers was in equilibrium with the aerated saline above them. The reason for these variations is that the coefficient of oxygen diffusion, in agar and gelatin, is very nearly the same and equal to that in saline, while in perspex the coefficient is essentially zero. Thus, the effective volume from which the electrode draws oxygen is reduced considerably when in contact with the perspex, resulting in a marked decrease in current. This simple experiment demonstrates conclusively the importance of the medium around the active tip of the electrode. It is obvious that in a heterogeneous medium such as tissue, which contains substances with widely varying coefficients of oxygen diffusion, the physical distribution of these substances alone will have a very great influence on the diffusion current, independent of any variation in the oxygen concentration. It is not difficult to see that in the extreme case, the diffusion current of a flat-ended electrode approaching heterogeneities, such as the walls of the larger blood vessels, bone, etc. (which have very small coefficients of oxygen diffusion) would decrease to almost zero and would not represent the oxygen concentration. No individual determination of diffusion current in tissue can be rigorously compared to another unless the physical distribution of the various tissue components around the active electrode tip is identical in the two cases.

If it is possible to fix the electrode within tissue then the relative value of the diffusion current, after a change in physiological conditions has been made, should represent a change in oxygen concentration. Whenever possible one should always verify the experimental readings by returning the tissue to its original state and observing whether the diffusion current can be reproduced. Also it should be pointed out that when the exposed electrode is being used to measure oxygen tension, which is changing with time, the current lags behind this change. Whether this becomes a major error depends on the rate at which the tension is changing. In Fig. 1, the curve corresponding to a time of 80 seconds is essentially the steady state condition in which the integral term of equation [2] has become negligible; it is clear that there may be many experimental situations where the steady state current will never be attained.

Discussion

A number of investigators have recently designed electrodes which operate satisfactorily in the presence of protein (9, 13, 14, 15). These authors employ one of a number of possible membranes to exclude the protein from the platinum surface and calomel cell, while permitting free exchange of oxygen. Such an assembly is ideal for measuring blood oxygen tension in vitro, or in the larger arteries and veins. Also, one of them (13) claims that the electrode will measure the concentration of oxygen in the surface layers of tissue. As mentioned previously the main reasons for not adopting such a modification were because such an electrode is large and because the time resolution is considerably increased. In addition, these membranes invariably become damaged when inserted into tissues, resulting in erroneous readings which cannot always be detected. Applying an appropriate correction to the diffusion current reading, and returning the exposed electrode to its original state by cleaning, as described, proved to be the most acceptable technique for routine experimentation.

Even after allowance has been made for the gradual decrease in the diffusion current observed in protein solutions, direct information about the dissolved oxygen is not always obtained. The main argument among persons using the exposed electrode is concerned with whether it responds to oxygen concentration or tension. This is a problem common to a number of other fields which appears to be somewhat academic (16). The electrode reaction is a dynamic process and depends on the number of molecules of oxygen arriving at its surface, that is, oxygen pressure. In most solutions one can easily calculate the oxygen concentration which bears a simple relation to pressure or tension, so the electrode can be said to respond to either. In solutions containing oxygen buffers, such as whole blood, where the concentration is a more complicated function of tension, a relation can still be established between diffusion current and either tension or concentration. The fact that our experimental data for whole blood yielded a linear relation between diffusion current and oxygen tension, for the range 40 to 150 mm Hg, supports

this argument. Although there must certainly be large numbers of red cells, 'oxygen sources', within the active volume of the electrode resulting in a high concentration of oxygen, none of this oxygen is being reduced.

A possible explanation is that oxyhaemoglobin, which liberates oxygen only in response to changes in the oxygen tension of the surrounding medium, acts as a carrier, rather than a source, once transient conditions have disappeared. The diffusion of oxygen would thus still be governed by the driving pressure from the bulk of the solution to the platinum surface, and not by the oxygen concentration. Considering the diffusion current in whole blood relative to plasma, one might even conclude that the red cells act more as physical barriers to oxygen diffusion than as 'oxygen sources'. Even with these limitations imposed the technique still can be used with considerable confidence for measuring the *relative* oxygen concentration. Based on these relative measurements a method for measuring tissue oxygen consumption has been developed (17).

Conclusions

1. The distribution of oxygen around the stationary platinum electrode, on applying an electrolyzing voltage, was calculated from the equation based on symmetrical spherical diffusion. It was thus demonstrated that the oxygen diffusion current of even the so-called microelectrode depends on conditions in a large volume of tissue surrounding the active electrode tip.

2. When measurements were made in protein solutions, it was found necessary to monitor the oxygen polarogram continuously by measuring the diffusion current at two different voltages.

3. A linear response between oxygen tension and electrode current was obtained with saline, blood, plasma, skin, and glycerin. The gradual decrease of the oxygen diffusion current when the electrode was used in protein solutions was reversed by a treatment with acid dichromate solution followed by neutralization with phosphate buffer.

4. By moving the electrode toward interfaces having different coefficients of oxygen diffusion, the volume of influence around the active tip of the electrode was demonstrated. Such an experiment indicates the marked effect which the surrounding medium will have on the electrode reaction.

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References

1. CONNELLY, C. M. Federation Proc. **16**, 681 (1957).
2. CHANCE, B. Federation Proc. **16**, 671 (1957).
3. HARRIS, E. D. and LINDSEY, A. J. Nature, **162**, 413 (1948).
4. DAVIES, P. W. and BRINK, F. Rev. Sci. Instr. **13**, 524 (1942).
5. MONTGOMERY, H. and HORVITZ, O. J. Clin. Invest. **29**, 1120 (1950).
6. CATER, D. B., PHILLIPS, A. F., and SILVER, I. A. Proc. Roy. Soc. **146**, 302 (1957).
7. URBACK, F. Proc. Soc. Exptl. Biol. Med. **92**, 644 (1956).
8. THOMLINSON, R. H. and GRAY, L. H. Brit. J. Cancer, **9**, 539 (1955).
9. CLARK, L. C., WOLF, W., GRANGER, D., and TAYLOR, Z. J. Appl. Physiol. **6**, 189 (1953).
10. LONGMUIR, I. S. Biochem. J. **57**, 81 (1954).
11. KOLTHOFF, I. M. and LINGANE, J. J. Polarography Interscience Publishers, Inc., New York. 1952.
12. JORDAN, J., ACKERMAN, E., and BERGEN, R. L. J. Am. Chem. Soc. **78**, 2979 (1956).
13. CLARK, L. C. Trans. Am. Soc. Art. Int. Org. **2**, 41 (1956).
14. KREUZER, F., WATSON, T. R., and BALL, J. M. J. Appl. Physiol. **12**, 65 (1958).
15. SPROULE, B. J., MILLER, W. F., CUSHING, I. E., and CHAPMAN, C. B. J. Appl. Physiol. **11**, 365 (1957).
16. MILLINGTON, R. J. Science, **122**, 1090 (1955).
17. INCH, W. R. Rev. can. biol. **14**, 258 (1955).

THE INFLUENCE OF THIOURACIL ON THE RESPONSES OF THE IMMATURE PULLET TO ESTROGEN, WITH REFERENCE TO OVIDUCT HYPERTROPHY, SERUM CALCIUM, AND LIVER COMPOSITION[1]

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Abstract

Thiouracil was given to sexually immature pullets at a level of 0.05% of the diet for 14 days before and also during treatment with 0.5 mg estradiol benzoate (ODB) or 1.0 mg ODB daily for 14 days. The thiouracil treatment did not modify the estrogen-induced hypercalcemia after 7 days, but after 14 days it enhanced significantly the blood calcium response to 1.0 mg ODB per day. The positive effects of thiouracil and of estrogen on liver weight per kg live weight were significant and additive. The comparatively low level of thiouracil used did not increase liver fat per kg live weight significantly, but it increased significantly the positive effect of estrogen on liver fat. The thiouracil treatment had no significant effect on liver deoxyribonucleic acid phosphorus (DNAP) but it increased liver pentose nucleic acid phosphorus (RNAP) per kg live weight significantly both in absence and presence of estrogen. The thiouracil treatment had no effect on the responses of the oviduct to estrogen.

In a second experiment the effects of (*a*) 1.0% thiouracil for 10 days before and also during a 14-day estrogen treatment of 1.0 mg ODB per day and of (*b*) 1.0% thiouracil given concurrently with 1.0 mg ODB per day were studied. Neither thiouracil treatment affected the response of the oviduct to estrogen. The other results confirmed previous observations.

In a third experiment, 1.5 mg ODB was given *every other day* for 14 days. When thiouracil at a level of 1% was given concurrently with the estrogen, it enhanced significantly the response of the oviduct. When 1% thiouracil was given also for 10 days before the estrogen, then it did not enhance the response of the oviduct. This experiment suggests that the conditions under which thiouracil will enhance estrogen-induced hypertrophy of the oviduct are narrowly defined. Other results were in general agreement with previous observations.

Introduction

An experiment has been reported in which 1% dietary thiouracil depressed estrogen-induced hypercalcemia of immature pullets but enhanced estrogen-induced hypertrophy of the oviduct (1). In subsequent experiments 0.75% thiouracil in the diet depressed estrogen-induced hypercalcemia but did not affect estrogen-induced hypertrophy of the oviduct, except in one experiment, where there was a slight depression (2). In further experiments it was found that 0.75% thiouracil in the diet may depress the hypercalcemia induced by 0.5 mg estradiol benzoate (ODB) per day while enhancing that induced by 1.0 mg ODB per day (3). In these experiments thiouracil did not affect the hypertrophy of the oviduct, and the results suggested that thiouracil would not have altered the level of serum calcium at some level of ODB intermediate between the levels actually used. No analogous interactions were detected in respect of effects on the liver; the positive effects of thiouracil and of estrogen on liver weight, liver protein, liver lipid, and liver pentose nucleic acid (RNAP) were additive.

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The treatments with estrogen in all foregoing studies were begun concurrently with the introduction of thiouracil into the diet, the levels of thiouracil were relatively high, and the thiouracil treatments of themselves gave big increases of liver weight, liver protein, and liver lipid.

Since thiouracil does not establish a hypothyroid condition immediately, whereas estrogen does effect serum calcium, etc. rather quickly, it was decided to study the effects of lower levels of thiouracil in an experiment where the drug would be administered for 14 days before the beginning of the estrogen treatment. It was hoped in this way to bring about a depression of thyroid activity while avoiding or limiting possible direct effects of high levels of the drug on liver composition. The present paper describes an experiment of this kind and two experiments designed to clarify points that arose in connection with the results of the first experiment.

Experimental

Experiment 1

General Technique

Fifty-six crossbred pullets (Rhode Island Red × Barred Rock) were placed in individual cages. The birds were 8 weeks of age at the beginning and 12 weeks of age at the end of the experiment. The basal diet was made up so that the iodine content would not be unduly high. Its composition was as follows: corn meal 25, ground wheat 27, finely ground oats 20, wheat shorts 5, soybean oil meal 18, dried brewers' yeast 5, salt (NaCl) 0.5, commercial dicalcium phosphate 2. To each 100 lb of this mixture was added 6 g manganese sulphate, 200,000 I.U. vitamin A, and 10,000 I.U. vitamin D_3. The vitamins were supplied as so-called 'dry' preparations in order to avoid the use of fish oils. Food consumption was controlled so that each bird might consume the same weight of food each day in order to avoid effects resulting from difference in food consumption.

The birds were assigned at random among eight groups each of seven pullets. Groups C0, C1, C2, and C3 received the basal diet. Groups T0, T1, T2, and T3 received the basal diet supplemented with 0.05% thiouracil. After a preliminary period of 14 days, groups C0 and T0 were killed, dissected, and analyzed as described below. During the succeeding experimental period of 14 days, groups C2 and T2 received 0.5 mg ODB (as 'Progynon B', Schering) per day by intramuscular injection, while groups C3 and T3 received 1.0 mg ODB per day. Groups C1 and C2 received injections of an equivalent volume of sesame oil solvent. The pullets remained in excellent condition throughout the experiment.

Analytical Methods

The birds were decapitated and bled. Ovaries, oviducts, thyroids, livers, and kidneys were removed and weighed. Serum protein was determined by precipitating the proteins from 3 ml serum with trichloroacetic acid and determining the protein (N×6.25) in the precipitate by the macro-Kjeldahl

method. Serum calcium was determined by the method of Halverson (4). Tissue powders were prepared from liver, as described elsewhere (5), and analyzed for RNAP and DNAP by the method of Schneider (6). Liver fat was determined by the titrimetric method of McCance and Shipp (7), and the results were calculated as tristearin and reported as 'fat'.

Experimental Results

Preliminary Period

The average results for groups C0 and T0 are presented in Table I in order to show the effects of 0.05% thiouracil given for 14 days before the estrogen treatment was begun. Thiouracil had not affected the live weight, weights of ovaries or oviducts, or the serum protein level during this preliminary period, but it had produced a moderate and highly significant hypertrophy of the thyroid and significant increases in liver weight and kidney weight. These results are similar qualitatively to those previously obtained with higher levels of thiouracil (3), and are in agreement with earlier reports by Schultz and Turner (8). Thiouracil had increased total liver DNAP and RNAP, though the increase in DNAP was not significant and the ratio RNAP:DNAP was not affected. These results were in accord with previous observations (2) on the effects of thiouracil on pullets given combined estrogen–

TABLE I

EXPERIMENT 1. EFFECT OF THIOURACIL ON SEXUALLY IMMATURE PULLETS. AVERAGE RESULTS FOR PRELIMINARY 14-DAY PERIOD. SEVEN PULLETS PER GROUP

	Group No.		
	C0	T0	
	Thiouracil in diet, %		Significance by "t" test ($P = 0.05$)
	Nil	0.05	
Live wt., initial, kg	0.82	0.85	N.S.
Live wt., 14 days, kg	1.02	1.09	N.S.
Food consumed, kg	0.66	0.66	—
Ovary, g	0.26	0.26	—
Oviduct, g	0.13	0.13	—
Thyroid, mg	70	165	**
Serum protein, g/100 ml	2.8	2.7	N.S.
Liver, g/kg live wt.	16.0	20.7	*
Liver fat, g/kg live wt.	0.50	0.73	*
Liver DNAP, mg/100 g	30.9	29.7	N.S.
mg/kg live wt.	5.0	6.2	N.S.
Liver RNAP, mg/100 g	76.5	74.7	N.S.
mg/kg live wt.	12.3	15.5	*
Ratio RNAP/DNAP	2.5	2.5	N.S.
Kidney, g/kg live wt.	5.3	6.0	*

androgen treatments. Similar effects of thiouracil on the liver RNA of male rats that received approximately the same amounts of food have been described recently by Guggenheim *et al.* (9). The results for the preliminary period demonstrated, therefore, that the thiouracil treatment was sufficient to bring about hypertrophy of the thyroid within 14 days but not such as to lead to excessive hypertrophy of the liver or to modify the ratio RNAP:DNAP.

Experimental Period

The average results for the experimental period proper, during which the estrogen treatments were administered, are presented in Table II. The results of an analysis of variance are indicated in Table II in accord with the following convention, viz.,

NS (non-significant)	$P < 0.05$,
*	$P > 0.05$ and < 0.01,
**	$P > 0.01$ and < 0.005,
***	$P > 0.005$.

(i) *Live weights.*—None of the differences attained significance. In considering this result it must be remembered that food consumption was kept as uniform as practicable.

(ii) *Ovary weights.*—An apparent depressant effect of ODB on ovary weight did not attain significance, and thiouracil was completely without effect.

(iii) *Oviduct weights.*—Estrogen increased oviduct weights to about the same extent as had been observed previously for similar treatments (1, 3). Neither the effect of thiouracil nor the interaction of thiouracil and estrogen attained significance. Wright *et al.* (3) found that thiouracil did not have any effect on oviduct response to estrogen in experiments where estrogen treatments were similar to those used in the present experiment.

(iv) *Thyroid weights.*—Thiouracil evoked a large and very highly significant hypertrophy of the thyroids. The seemingly additional effect of estrogen did not attain significance except in so far as a "*t*" test on group T1 *vs.* group T3 gave "*t*" = 2.12 (2.18 required for $P = 0.05$ at 12 d.f.). Comparison of the data with those for the preliminary period (see Table I) shows that most of the hypertrophy took place during the second fortnight of the experiment.

(v) *Serum calcium and protein levels.*—After 7 days of estrogen treatment, both levels of estrogen had given significant increases of serum calcium, but thiouracil had not modified these responses significantly. After 14 days of estrogen treatment, the higher level of estrogen had produced a further increase of serum calcium, and the estrogen-induced hypercalcemia had been enhanced significantly by the thiouracil treatment. The results for serum protein resembled those for serum calcium, but the differences, other than those due to estrogen, did not attain significance.

(vi) *Liver weights and liver fat.*—Estrogen increased liver weight per kg live weight to a highly significant degree, both in presence and absence of thiouracil. Thiouracil also increased liver weight significantly, but the

TABLE II

EXPERIMENT 1. EFFECTS OF THIOURACIL ON RESPONSES OF SEXUALLY IMMATURE PULLETS TO ESTROGEN. FINAL AVERAGE RESULTS. SEVEN PULLETS PER GROUP

	Group No.						Source of variance and significance		
	C1	C2	C3	T1	T2	T3	Thiouracil vs. no thiouracil	Levels of estrogen	Interaction of thiouracil and estrogen
Estradiol benzoate, mg per day	Nil	0.5	1.0	Nil	0.5	1.0			
Thiouracil in diet, %*	Nil	Nil	Nil	0.05	0.05	0.05			
Live wt., initial, kg	1.07	1.06	1.04	1.06	1.06	1.06	N.S.	N.S.	N.S.
Live wt., final, kg	1.31	1.27	1.23	1.29	1.27	1.27	N.S.	N.S.	N.S.
Food consumed, 1–28 days, kg	1.72	1.73	1.74	1.71	1.75	1.74	N.S.	N.S.	N.S.
Ovary, g	0.26	0.25	0.24	0.30	0.23	0.23	N.S.	N.S.	N.S.
Oviduct, g	0.16	7.1	13.4	0.17	6.3	12.0	N.S.	***	N.S.
Thyroid, mg	79	82	97	388	471	511	***	N.S.	N.S.
Serum calcium, mg/100 ml, 7 days estrogen	12.8	22.8	45.9	12.8	26.9	48.1	N.S.	***	N.S.
Serum calcium, mg/100 ml, 14 days estrogen	13.0	26.4	64.1	12.6	23.6	79.1	N.S.	***	**
Serum protein, g/100 ml, 14 days estrogen	2.8	3.9	5.8	2.9	3.5	6.1	N.S.	***	N.S.
Liver, g/kg live wt.	15.2	18.8	24.5	17.3	20.5	27.2	*	***	N.S.
Liver fat, g/kg live wt.	0.51	1.04	1.33	0.58	0.96	1.76	N.S.	***	***
Liver DNAP, mg/100 g liver	30.7	27.9	22.7	28.8	25.0	22.0	N.S.	***	***
mg/kg live wt.	4.7	5.2	5.6	5.0	5.1	6.0	N.S.	**	N.S.
Liver RNAP, mg/100 g liver	77.9	80.2	81.3	76.1	77.6	82.8	N.S.	***	N.S.
mg/kg live wt.	12.4	15.0	19.9	13.2	15.9	22.5	*	***	N.S.
Ratio RNAP:DNAP	2.6	2.9	3.6	2.7	3.1	3.8	***	***	N.S.
Kidney, g/kg live wt.	5.1	5.3	5.8	5.7	5.7	6.8	***	***	N.S.

*Thiouracil treatment begun 14 days before beginning of 14-day period of estrogen treatment.

effect was smaller than that observed previously with higher levels of thiouracil (3). The effects of estrogen and thiouracil were additive, as shown by the non-significance of the interaction. Liver fat per kg live weight was increased to a highly significant degree by estrogen though not by thiouracil. However, thiouracil greatly enhanced this effect of estrogen, as shown by the high significance of their interaction.

(vii) *Liver nucleic acids.*—Liver DNAP per kg live weight was increased slightly though highly significantly by estrogen, in agreement with previous observations. (Since the livers were not exsanguinated by perfusion, this effect may reflect hyperplasia and/or a greater residue of red cells in livers of estrogenized birds.) Thiouracil had no effect on liver DNAP per kg live weight nor was its interaction with estrogen significant. Liver RNAP per kg live weight was increased highly significantly by estrogen and significantly by thiouracil. The effect of thiouracil was a consequence mainly of the increase in liver weight, and in this respect resembled a similar effect reported for the rat by Guggenheim *et al.* (9). There was no significant interaction of these effects of estrogen and thiouracil, so that their effects on liver RNAP were additive. The ratio RNAP:DNAP was increased to a highly significant degree by estrogen and by thiouracil and again there was no interaction.

(viii) *Kidney weights.*—Estrogen increased kidney weight highly significantly, in confirmation of previous experiments. Thiouracil increased kidney weight to a very highly significant degree, confirming previous results. The absence of any significant interaction shows that these effects of estrogen and of thiouracil on kidney weight were additive, as in the case of liver.

Experiment 2

The results of experiment 1 were in general accord with previous observations (2, 3). However, it seemed desirable to investigate further the possible effect of thiouracil on estrogen-induced hypertrophy of the oviduct that had been observed in our previous experiment (1). Accordingly, the effect of 0.05% thiouracil introduced 10 days before the beginning of the estrogen treatment was compared with the effect of 1% thiouracil.

The experimental birds comprised 32 crossbred pullets (Rhode Island Red × Barred Rock) which were 10 weeks of age at the outset of the experiment. The general experimental technique, the basal diet, and the analytical methods were the same as in experiment 1, except that no birds were killed at the end of the preliminary period and liver nucleic acid phosphorus was not determined.

Experimental Results

The results of experiment 2 are presented in Table III.

(i) *Live weights.*—No significant differences between groups were observed.

(ii) *Ovary weight.*—The depressant effect of estrogen on ovary weight attained significance in this experiment. Thiouracil did not affect ovary weight.

TABLE III

EXPERIMENT 2. EFFECTS OF THIOURACIL ON THE RESPONSES OF SEXUALLY IMMATURE PULLETS TO ESTROGEN. AVERAGE RESULTS. EIGHT PULLETS PER GROUP

	Group				
	1	2	3	4	
Estradiol benzoate, mg per day	Nil	1.0	1.0	1.0	L.S.D.*
Thiouracil in diet, %	Nil	Nil	0.05†	1.00‡	$P = 0.05$
Live wt., initial, kg	0.99	0.98	0.99	0.97	N.S.
Live wt., final, kg	1.42	1.35	1.42	1.36	N.S.
Food consumed, days 1–24, kg	1.73	1.75	1.76	1.75	N.S.
Ovary, g	0.29	0.22	0.22	0.22	0.013
Oviduct, g	0.21	13.7	13.6	14.0	1.70
Thyroid, mg	90	97	310	89	57
Serum calcium, mg/100 ml, 3 days estrogen	12.6	25.1	21.7	21.3	3.30
Serum calcium, mg/100 ml, 14 days estrogen	13.9	72.4	82.7	79.3	12.9
Serum protein, g/100 ml, 14 days estrogen	2.9	6.3	5.8	5.8	0.82
Liver, g/kg live wt.	15.7	24.2	23.4	32.4	3.90
Liver crude protein, g/kg live wt.	3.25	4.17	4.27	5.55	0.71
Liver fat, g/kg live wt.	0.54	1.46	1.31	1.53	0.41
Kidney, g/kg live wt.	4.8	5.7	6.0	7.8	0.74

*L.S.D. denotes least significant difference between any two groups by the "F" test.
†Thiouracil treatment begun 10 days before estrogen.
‡Thiouracil begun only from start of estrogen treatment.

(iii) *Thyroid weight.*—Estrogen did not affect thyroid weight any more than in experiment 1. The 0.05% level of thiouracil, given over the preliminary 10 days as well as the period of estrogen treatment, produced a very highly significant hypertrophy of the thyroids of group 3. The 1.0% level, given only during the 14 days of estrogen treatment, did not enlarge the thyroids; this observation agrees with the relatively small degree of hypertrophy observed by Common *et al.* (2) as a result of treatment with 0.75% thiouracil given concurrently with a 14-day period of estrogen treatment.

(iv) *Oviduct weights.*—The hypertrophy of the oviducts induced by 1.0 mg ODB daily for 14 days was not affected by either thiouracil treatment. This conformed with experiment 1 and with previous observations so far as group 3 was concerned, but the result with group 4 was at variance with the observation of Common *et al.* (1).

(v) *Serum calcium and protein.*—After 3 days, both the thiouracil treatments tended to depress the effect of estrogen on serum calcium. By the end of the experiment, however, thiouracil tended to enhance estrogen-induced hypercalcemia, although the effect of the 0.05 level of thiouracil attained significance only at $P = 0.10$. These results conform in general with the similar observations of Wright *et al.* (3).

(vi) *Liver weight, liver protein, and liver fat.*—Estrogen produced the usual increases of liver weight, liver protein per kg live weight, and liver fat per kg live weight. The treatment with 0.05% thiouracil did not affect significantly these responses, but the treatment with 1% thiouracil gave further increases.

(vii) *Kidney weight.*—The results for kidney weight per kg live weight fully confirmed that the estrogen-induced increase of kidney weight is increased slightly by low levels of thiouracil and to a marked degree by 1.0% thiouracil given concurrently with the estrogen.

Experiment 3

At this stage it seemed that enhancement by thiouracil of estrogen-induced hypertrophy of the oviduct is not likely to be observed under the following conditions:

(*a*) Thiouracil at 1% of diet and begun concurrently with estrogen treatment; 0.5 mg or 1.0 mg ODB daily for 14 days.

(*b*) Thiouracil at 0.05% of diet but given for 14 days before as well as during estrogen treatment; 0.5 mg or 1.0 mg ODB daily. It was decided, therefore, to seek confirmation of the original experiment (1) by repeating it as closely as possible. The estrogen treatment was 1.5 mg ODB *every other day* over a 14-day period, and the thiouracil treatment consisted of the introduction of 1% thiouracil into the diet at the start of the estrogen treatment. An extra group (group 4) was included in which the 1% thiouracil was begun 10 days before the estrogen.

The experimental birds comprised 32 crossbred pullets (Rhode Island Red × Barred Rock). The birds were 15 weeks of age at the end of the experiment. The experiment was performed similarly to experiment 2, except that a commercial 'starter' mixture was used as the basal ration.

Experimental Results

The experimental results are set out in Table IV.

(i) *Live weights.*—None of the differences between groups were statistically significant.

(ii) *Ovary weight.*—The tendency of estrogen to depress ovary weight attained significance in so far as a comparison of groups 1 and 2 by the "*t*" test was concerned ("*t*" = 2.75 for 14 d.f.). The effects of thiouracil on the responses to estrogen were not significant.

(iii) *Oviduct weight.*—The estrogen treatment produced a hypertrophy of the expected magnitude. One per cent thiouracil given concurrently with the estrogen produced a marked and significant enhancement of this response to estrogen. This confirmed the observation of Common *et al.* (1) on this point.

(iv) *Thyroid weights.*—None of the differences of thyroid weight attained significance. The absence of an effect in group 3 was not astonishing in view

TABLE IV

EXPERIMENT 3. EFFECTS OF THIOURACIL ON THE RESPONSES OF THE SEXUALLY IMMATURE PULLET TO ESTROGEN. AVERAGE RESULTS. EIGHT PULLETS PER GROUP. ESTROGEN GIVEN OVER A 14-DAY PERIOD

	Group				
	1	2	3	4	
Estradiol benzoate, mg *every other day*	Nil	1.5	1.5	1.5	L.S.D.*
Thiouracil in diet, %	Nil	Nil	1.00†	1.00‡	$P = 0.05$
Live wt., initial, kg	1.02	1.01	0.96	0.97	N.S.
Live wt., final, kg	1.36	1.33	1.32	1.32	N.S.
Food consumed, days 11–24, kg	1.66	1.68	1.68	1.68	N.S.
Ovary, g	0.32	0.24	0.25	0.28	N.S.
Oviduct, g	0.18	10.9	15.6	10.8	3.5
Thyroid, mg	159	139	110	211	N.S.
Serum calcium, mg/100 ml	14.7	54.4	52.1	24.7	18.2
Serum protein, g/100 ml	3.3	5.2	4.7	3.9	0.98
Liver, g/kg live wt.	15.4	20.8	29.9	43.3	6.69
Liver crude protein, g/kg live wt.	3.39	4.22	5.66	7.07	0.79
Liver fat, g/kg live wt.	0.58	0.90	1.29	1.18	0.24
Kidney, g/kg live wt.	5.6	5.8	8.2	8.5	0.65

*L.S.D. denotes least significant difference between any two goups by the "*F*" test.
†Thiouracil treatment begun at the same time as estrogen.
‡Thiouracil treatment begun 10 days before estrogen.

of the relatively small effect observed with similar treatments in other experiments, but group 4 might have been expected to display a greater hypertrophy since thiouracil was given over a total of 24 days. The use of a commercial feed mixture may have played a part here; such mixtures frequently contain some form of iodine supplement.

(v) *Serum calcium and protein.*—The serum calcium in the control group was higher than is usual in sexually immature birds at this age, but the ovaries did not display any sign of the approach of reproductive activity. Estrogen gave the usual large increase in serum calcium level. Contrary to the earlier findings on this point (1), thiouracil treatment of group 3 did not depress the serum calcium level, although a depression was observed when the thiouracil was given also for 10 days before the estrogen. Thiouracil did not depress significantly the serum protein level in group 3, but it did depress the serum protein in group 4, where the thiouracil treatment was begun before the estrogen treatment.

(vi) *Liver weight, liver protein, and liver fat.*—The results for liver weight followed the expected pattern. The magnitudes of the responses conformed rather closely with those previously observed (1) for the same treatment.

(vii) *Kidney weight.*—A slight but non-significant increase due to estrogen was probably real, since estrogen increased kidney weight in experiments 1 and 2. The thiouracil treatments gave a big increase of kidney weight.

Discussion

The present experiments support the conclusion that the effects of thiouracil on responses to estrogen are less complex as regards liver and kidney than as regards serum calcium, serum protein, and hypertrophy of the oviduct. It is well established that estrogen increases liver weight, total liver protein, total liver lipid, total liver RNAP, and the ratio RNAP:DNAP in liver. It is reasonably clear also that thiouracil of itself produces similar effects even when given at the level of 0.05% (experiment 1). These effects of estrogen and thiouracil are additive in the chicken, for no significant interaction between them has been detected except in relation to liver lipid, where a low level of thiouracil that did not of itself increase liver lipid nevertheless did augment the positive effect of estrogen on liver lipid (experiment 1).

Estrogen increases kidney weight. Thiouracil also increases kidney weight, the effect being great with high levels of thiouracil. Here again the effects of thiouracil and of estrogen are additive.

None of the differences in duration and level of dosage that have been studied have affected the results in respect of the foregoing effects other than in quantitative senses that may reasonably be ascribed to level and duration of treatment.

The situation as regards hypertrophy of the oviduct, hypercalcemia, and hyperproteinemia is more complicated. Thiouracil administered to pullets treated with 0.5 mg or 1.0 mg ODB per day has not altered the response of the oviduct to estrogen (2, 3, experiment 1) apart from the slight depression noted in one experiment (2), nor has it made any difference whether the thiouracil was given at a level of 1.0% beginning with the estrogen treatment or 0.05% beginning 14 days before the estrogen treatment. However, when estrogen was given at 1.5 mg ODB *every other day* over a 14-day period, then 1% thiouracil begun at the same time as the estrogen enhanced the response of the oviduct (1), and this observation has now been confirmed by the results of experiment 3. It was originally suggested that this effect might be due to impairment of liver function by estrogen, with consequent impaired inactivation of estrogen (1). The present experiments have provided two pieces of evidence that are at variance with this suggestion. Firstly, if impairment of liver function is responsible, then 1% thiouracil given for 10 days before estrogenization might be expected to impair liver function even more, and to give at least as great an enhancement of hypertrophy. In point of fact this has been found not to be so, for in experiment 3 the treatment in question did not affect oviduct hypertrophy. Secondly, one might surmise that impairment of liver function would result in decreased phosphoprotein synthesis and in lowered serum calcium response to estrogen as well as in enhanced oviduct hypertrophy. In experiment 3 no such depression of serum calcium was observed in the group that displayed enhancement of the hypertrophy, although it is true that it was evident in the group that received 1% thiouracil for 10 days before estrogen. It remains obscure why thiouracil may enhance estrogen-induced hypertrophy of the oviduct, even

though only under rather closely defined conditions that include dosage every other day instead of every day. The available evidence suggests that the effect is not a simple consequence of a hypothyroid condition; indeed, there is evidence (10, 11, 2) that thyroxine has little effect on estrogen-induced hypertrophy of the oviduct. There remains the possibility that dietary levels of iodine may be implicated, since commercial starter rations were used in the two experiments where the enhancing effect of thiouracil on the oviduct hypertrophy was observed, but this matter can only be settled by further experimentation.

Thyroxine is known to depress estrogen-induced hypercalcemia (10, 11, 2) and a hypothyroid condition induced by thiouracil might be expected, a priori, to have an opposite effect. However, when thiouracil is given at a high level beginning with the estrogen treatment, then over the first week or so it may actually depress the response of serum calcium to 0.5 mg ODB daily while having little effect at 14 days (3). This might be interpreted as a consequence of transient stimulation of thyroid activity by thiouracil. At a higher level of ODB (1.0 mg daily) thiouracil had little effect on serum calcium at first, but it may enhance serum calcium at 14 days. This observation may be related to the effects of estrogen itself on thyroid activity. Noach (12) has pointed out that estrogen may affect thyroid activity either by inhibiting release of thyrotrophin or by potentiating the action of TSH on the thyroid, and that the net effect is probably a summation of these opposing actions. No data are available, however, to show in what way the actual estrogen treatments used in the present experiments may have affected thyroid activity. However, in experiment 1, where 0.05% dietary thiouracil was introduced 14 days before the estrogen treatments, the data for thyroid hypertrophy and the absence of a significant effect of thiouracil on liver fat suggest that a relatively uncomplicated hypothyroid condition had supervened by the end of the experiment. If this be granted, then the augmentation of estrogen-induced hypercalcemia by thiouracil in experiment 1 may be interpreted as a direct consequence of thyroid insufficiency. The evidence from experiment 2 is in accord with this tentative hypothesis, for the depressant effect of both thiouracil treatments on serum calcium after 3 days of estrogen treatment could then be attributed to an initial stimulation of thyroid activity, while the tendency to an enhancing effect of thiouracil on serum calcium by the end of the experiment would accord with establishment of thyroid insufficiency by that time. In experiment 3, 1% thiouracil begun with the estrogen had little effect on the hypercalcemia by the end of the experiment, in accord again with the foregoing argument. The depressant effect of 1% thiouracil on the hypercalcemia in experiment 3 when begun 10 days before the estrogen treatment is less readily explicable; it may be that the prolonged treatment with this high level of thiouracil had impaired liver function so far as production of serum protein was concerned. Certainly this prolonged treatment with 1% thiouracil had increased very greatly the size and fat content of the liver.

Guggenheim *et al.* (9) have noted that thiouracil (0.5% of the diet) increased the liver weight of rats more than did thyroidectomy, so that the effects of thiouracil, or at least of high levels of thiouracil, on the liver were not identical with those of hyperthyroidism in their experiments.

The following working hypothesis may be advanced as a tentative explanation of the results of the foregoing observations: When thiouracil is given at a high level (0.5 to 1% of the diet) beginning with the estrogen treatment, then the initial outcome is a transient stimulation of thyroidal activity. This will result in a lower hypercalcemia response to lower levels of estrogen, though the effect may not make itself apparent at higher levels of estrogen (i.e. 1.0 mg per day), a circumstance that may be related to the effect of the estrogen on thyroid activity. Continuation of the thiouracil treatment, however, will eventually bring about a hypothyroidal state, and thiouracil will then enhance the hypercalcemic response because of the depression of thyroidal activity. In the same way a low level of thiouracil given for a time before beginning the estrogen treatment may also increase the hypercalcemic response. With high and prolonged treatment with thiouracil, the production of serum proteins, especially of serum phosphoproteins, by the liver may be impaired by effects of thiouracil other than those due to its antithyroidal action, so that estrogen-induced hypercalcemia and hyperproteinemia will be depressed thereby.

Conclusions

The results of the present work, together with those of previous experiments, permit of the conclusion that thiouracil of itself increases liver weight, total liver protein, total liver RNAP, and the ratio RNAP:DNAP in liver. Estrogen of itself produces similar effects. The present results support the conclusion that the effects of estrogen and thiouracil are additive in the foregoing respects. Previous work has shown that high levels of thiouracil will increase liver lipid greatly and that this effect is additive with the similar positive effect of estrogen. The present work has shown that a level of thiouracil too low of itself to increase liver lipid significantly may nevertheless augment the positive effect of estrogen on liver lipid (as in experiment 1).

It may also be concluded that thiouracil does not in general affect the response of the oviduct to estrogen; but with the reservation that a high level of thiouracil under certain narrowly defined conditions, including dosage every other day instead of every day, may enhance the response. The reasons for this latter observation remain obscure, but the available evidence does not suggest any simple explanation on grounds of the effects of thiouracil on thyroidal activity.

The effects of thiouracil on estrogen-induced hypercalcemia and hyperproteinemia may be positive, or negative, or inappreciable, depending on the level of thiouracil, the duration of thiouracil treatment, the level of estrogen treatment, and possibly on other factors as well.

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References

1. COMMON, R. H., KEEFE, T. J., and MAW, W. A. Can. J. Research, D, **28**, 272 (1950).
2. COMMON, R. H., MAW, W. A., and LAYNE, D. S. Can. J. Biochem. and Physiol. **33**, 667 (1955).
3. WRIGHT, L. A., MAW, W. A., and COMMON, R. H. Can J. Biochem. and Physiol. **34**, 817 (1956).
4. PETERS, J. P. and VAN SLYKE, D. D. Quantitative clinical chemistry. Vol. 2. Baillière, Tindall & Cox, Ltd., London, 1932.
5. LAYNE, D. S. and COMMON, R. H. Can. J. Biochem. and Physiol. **33**, 517 (1955).
6. SCHNEIDER, W. C. J. Biol. Chem. **161**, 293 (1945).
7. MCCANCE, R. A. and SHIPP, H. L. The chemistry of flesh foods and their losses on cooking. Med. Research Council Special Report Series No. 187. His Majesty's Stationery Office, London. 1933.
8. SCHULTZ, A. B. and TURNER, C. W. Univ. Montana Agri. Exptl. Sta. Research Bull. **392**, 1 (1945).
9. GUGGENHEIM, K., HALEVY, S., SINGER, D., and USIELI, V. Endocrinology, **62**, 355 (1958).
10. FLEISCHMANN, W. and FRIED, L. A. Endocrinology, **36**, 406 (1945).
11. COMMON, R. H., RUTLEDGE, W. A., and BOLTON, W. J. Endocrinol. **5**, 263 (1948).
12. NOACH, E. L. Acta Endocrinol. **19**, 127 (1955).

STUDIES OF LIGNIN BIOSYNTHESIS USING ISOTOPIC CARBON

VI. FORMATION OF THE SIDE CHAIN OF THE PHENYLPROPANE MONOMER[1]

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Abstract

A number of aromatic compounds having carbon side chains of varying length and substitution have been studied as lignin precursors. *p*-Hydroxybenzaldehyde was poorly utilized, indicating that the formation of C_6,C_3 units from a condensation of C_6,C_1, and C_2 fragments is of very limited significance. The C_6,C_2 compound, mandelic acid, was not converted to lignin. DL-Phenyllactic acid was incorporated into lignin with much lower dilution than either DL-*erythro*- or DL-*threo*-phenylglyceric acids, and with appreciably lower dilution than DL-phenylhydracrylic acid, supporting the concept that lignin monomers are formed via 2-hydroxylated phenylpropane intermediates. Wheat utilized the D-form of phenylalanine less readily than the L-form. Although three monocotyledons tested could utilize (+)- and (−)-phenyllactic acid to a comparable degree, two dicotyledons showed a marked preference for the (−)-form, suggesting that in dicotyledons the enzyme system specific for the (−)-form is predominant.

Introduction

Present theories of lignin formation involve the polymerization of radicals of the semiquinone type derived from *p*-coumaryl, coniferyl, and sinapyl alcohols. The resultant quinone methine intermediates are then stabilized by the addition of an alkoxy-, phenoxy-, or hydroxy- group at the 3-position of the side chain, and thus the well established secondary lignin building units are obtained (1, 2, 3). However, the individual steps by which the above monomers are synthesized from carbon dioxide by the plant have yet to be established. In this laboratory it has been shown that a wide variety of C_6,C_3 compounds which can be envisaged as being readily metabolized by simple processes of oxidation, reduction, and dehydration into cinnamyl residues were quite readily incorporated into lignin, although in the case of tyrosine marked species differences have been reported (4, 5). From the relative efficiencies of incorporation of the various C_6,C_3 compounds some of the metabolic pathways have been tentatively suggested. While it has been firmly established that the shikimic acid pathway to the aromatic ring is operative in the biosynthesis of lignin (6, 7), other pathways cannot be ruled out. Thus it was demonstrated that several C_6,C_1 compounds showed moderate efficiency as lignin precursors (4). Moreover, the alternative route to the aromatic ring by the head-to-tail condensation of acetate units (8) remains possible. However, Watkin, Underhill, and Neish (9) showed that essentially all the radioactivity incorporated into quercetin on the feeding of acetate-C^{14} to buckwheat was located in the phloroglucinol-type ring and that the remaining nine carbons, corresponding to a phenylpropane

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unit, arise via the shikimic acid pathway. In the present study a further series of possible lignin precursors has been fed to several plant species. These precursors have included a number in which the structure of the side chain has been varied, both as regards position and number of hydroxyl functions and number of carbon atoms. Data from these experiments have provided further clues to the origin of the side chain of the lignin monomers.

Experimental

Cultivation of the Plants

Wheat (*Triticum vulgare* Vill. var. Thatcher) and buckwheat (*Fagopyrum tataricum* Gaertn. var. C. D. 4251) were grown in soil out of doors, or in gravel culture under artificial light as previously described (4). *Salvia splendens* Sello var. Globe of Fire also was grown in gravel culture. Maple (*Acer negundo* L. var. *interius* Sarg.), sedge (*Carex atherodes* Spreng.), and spangletop grass (*Fluminia festucacea* Hitchc.) were collected from the wild state. All plants except the maple were used in the early flowering stage.

Synthesis of C^{14}-Labelled Compounds

C^{14}-Labelled sodium carbonate and randomly labelled samples of benzaldehyde and L-phenylalanine were purchased from Atomic Energy of Canada. The preparation of *p*-hydroxybenzaldehyde, carbonyl-C^{14}, is described in a previous paper of this series (5).

DL-Mandelic acid, labelled in all carbons except the carboxyl carbon, was prepared from randomly labelled benzaldehyde and unlabelled potassium cyanide (10).

Part of the randomly labelled benzaldehyde was converted to cinnamic acid (84% yield) by reaction with malonic acid under the conditions used by Koo *et al.* (11) for 2,3-dimethoxybenzaldehyde. The *cinnamic acid-ring, 3-C^{14}*, thus obtained (0.65 g) was dissolved in 3 ml of glacial acetic acid containing 30% HBr and the mixture allowed to stand 3 days at room temperature in a 25 ml glass-stoppered conical flask. Most of the acetic and hydrobromic acid was evaporated by an air stream at room temperature. The residue was dissolved in 10 ml of hot benzene and evaporated to dryness. This gave 1.04 g of a crude solid which was recrystallized from benzene, giving 0.78 g (74%) of 3-phenyl-3-bromopropionic acid, labelled in the ring and carbon-3. This was boiled 15 minutes with 8 ml of water, cooled, filtered, and the filtrate extracted with four 25-ml portions of ether. The ether was evaporated and the residue recrystallized twice from chloroform to give 0.37 g of DL-*phenylhydracrylic acid-ring,3-C^{14}*, m.p. 92–93° C. The over-all yield based on cinnamic acid was 49%. This modification of the procedure of Fittig and Binder (12) gave better yields than that described by La Mer and Greenspan (13).

Carboxyl-labelled benzoic acid, prepared by carbonation of phenyl magnesium bromide, was converted to benzoyl chloride by treatment with thionyl chloride. The benzoyl chloride was purified by distillation (6.75 mM.; yield 80%) and reduced by Rosenmund's method in 4 ml of xylene observing

the precautions described previously (5). The reduction was completed in 2 hours; the catalyst was filtered out and washed with 10 ml of dry pyridine. The combined filtrate and washings was refluxed 4 hours with 1.7 g of malonic acid and 2 ml of dry piperidine (11). This gave 0.73 g (75% based on benzoyl chloride) of *cinnamic acid-3-C^{14}*, m.p. 133° C.

Cinnamic acid-3-C^{14} was converted to DL-*erythro*-phenylglyceric acid-3-C^{14} through the chlorohydrin as described by Boeseken (14). The hypochlorous acid used was prepared as described by Chung and Israel (15). The cinnamic acid (1.48 g) was neutralized with 22.5 ml of 0.455 *N* KOH and this solution treated with 7.0 ml of 1.43 *M* HOCl at 3° C. The temperature rose at once to about 17° C. After 30 minutes at room temperature the reaction mixture was treated as described previously (14) to give 0.57 g of DL-erythro-*phenylglyceric acid-3-C^{14}*, m.p. 121–122° C, purified by recrystallization from ether. The yield was 31% based on cinnamic acid.

Methyl cinnamate-3-C^{14} was made by treating cinnamic acid (0.74 g) with excess diazomethane in ether. This ester was treated with permanganate, essentially as described by Riiber (16). The product was isolated by continuous extraction with ether and purified by washing with ether. This gave 0.48 g (53%) of colorless crystals of DL-threo-*phenylglyceric acid-3-C^{14}*, m.p. 141–142° C.

DL-Phenylalanine-3-C^{14} was prepared from benzaldehyde and 2-mercaptothiazole-5-one as described by Billimoria and Cook (17). Benzoyl chloride-carbonyl-C^{14} (1.07 g) was reduced in 4 ml of xylene by Rosenmund's method and the catalyst was filtered out and washed with 10 ml of glacial acetic acid. The combined filtrate and washings on being treated with 0.90 g of 2-mercaptothiazole-5-one and 0.02 ml of dry piperidine gave 1.05 g of crystalline 2-thio-4-benzylidenethiazolid-5-one. This was treated with red phosphorus and hydriodic acid as described (17) to give 0.58 g of DL-*phenylalanine-3-C^{14}* as shining colorless plates. The yield was 46% based on the benzoyl chloride.

Phenylalanine-3-C^{14} was resolved into the D- and L-forms through the fenchylamine salts of N-acetyl phenylalanine as described by Overby and Ingersoll (18). The L-phenylalanine-3-C^{14} thus obtained had an optical purity of at least 98% while the D-isomer had a purity of at least 96%, calculated from the optical rotations.

A sample of DL-phenylalanine, prepared from randomly labelled benzaldehyde and 2-mercaptothiazole-5-one (17), was deaminated to DL-phenyllactic acid by nitrous acid. Deamination at low temperature (19) gave a poor yield; better results were obtained at a higher temperature as used by Bredereck *et al.* in deamination of guanosine (20). DL-Phenylalanine (1.0 g) was dissolved in 12 ml of 2 *N* sulphuric acid. The mixture was heated to 75–80° C. and stirred while a solution of 1.2 g of sodium nitrite in 15 ml of water was added gradually during 2 hours. The reaction mixture was cooled and extracted continuously with ether. The extract was evaporated and the residue taken up in hot water and filtered through Celite. The filtrate

was evaporated to 2–3 ml. On cooling at 3° C DL-*phenyllactic acid-ring, 3-C*^{14}, crystallized out; 0.49 g m.p. 96.5–97.5° C. A second crop 0.16 g m.p. 94–96° C was obtained from the mother liquors. This gave a total yield of 64%.

DL-Phenyllactic acid was resolved by strychnine as described by Mori (21). The (+)-phenyllactic acid-ring,3-C^{14}, had m.p. 125° C, $[\alpha]_D^{24} = 21.6°$, $c = 1$, water, while the (−)-isomer, m.p. 124–5° C, had $[\alpha]_D^{24} = -21.2°$, $c = 1$, water.

Administration of Labelled Compounds

D- and L-phenylalanine, and the sodium salts of *p*-hydroxybenzaldehyde and the organic acids were administered through the cut ends of the stems from water essentially as described earlier (5). About 5 μc of carbon-14 was used in each administration, and the compounds were given in *ca.* 0.04 *M* solution. After the absorption of the radioactive solution, the plants were allowed to metabolize in a chamber illuminated by fluorescent tubes with a light intensity of *ca.* 5000 lumens/m^2 (500 ft-c) for approximately 24 hours at 21° C.

Isolation and Counting of Lignin and Its Derivatives

The preparation and oxidation of the cell wall fraction and the isolation and purification of the phenolic aldehydes were done as previously reported. The aldehydes were separated on columns of diatomaceous earth, and converted to their *m*-nitrobenzoylhydrazones which were combusted to carbon dioxide for C^{14}-analysis (4).

Results and Discussion

The results of earlier studies in this series have indicated that lignin arises from aromatic compounds bearing a three-carbon side chain. The plant, at least in some circumstances, finds it necessary to remove the 3-, 4-, and 5-hydroxyl groups from shikimic acid in the process of aromatization, and the resultant phenylpyruvic acid is hydroxylated (probably by phenolase) to yield eventually the appropriately substituted cinnamyl alcohols. That these suffer relatively little further substitution, once polymerized, is indicated by the fact that ferulic acid can be preferentially converted to guaiacyl lignin much more readily than to syringyl lignin. It also appears likely from these experiments that unsaturation is introduced into the side chain before reduction of the carboxyl group. The present studies were undertaken in an effort to gain information about the reactions involved in the formation of the side chain of the lignin monomer, and to delineate at least in part the transformation which plants can bring about in the structure and substitution of the side chain.

Because of the high efficiency with which shikimic acid is utilized in lignification (6) it has been assumed, by analogy to known reactions in microorganisms, that the normal pathway involves the addition of a three-carbon side chain at the shikimic acid stage. However, as it has been shown in this laboratory that vanillin and *p*-hydroxybenzoic acid can be incorporated into

lignin to a limited extent (4), it was considered desirable to investigate further the suggestion that existing side chains can be lengthened after aromatization.

A mechanism for the conversion of C_6,C_1 compounds to the phenylpropane type was discovered by Neuberg and Hirsch (22), who showed that fermenting yeast could condense benzaldehyde with acetaldehyde to give phenylacetylcarbinol and hence methylphenylglyoxal. Higuchi, Ito, and Kawamura (23) more recently found *p*-hydroxybenzaldehyde and vanillin to react similarly giving methyl *p*-hydroxyphenylglyoxal and methylguaiacylglyoxal, respectively, which might well be lignin precursors in view of the ease with which phenylpropane compounds with side chains at varying oxidation levels can be utilized. Although the earlier findings with vanillin were consistent with the existence of such a reaction in plants, Table I shows that *p*-hydroxybenzaldehyde is a very poor precursor of guaiacyl and syringyl lignin in wheat, much poorer than vanillin (4), again indicating the very limited extent to which condensations of the $C_6,C_1 + C_2$ type are operative in this species. It is not considered that too much significance should be attached at present to the very low dilution of carbon-14 in the *p*-hydroxybenzaldehyde isolated in this experiment. The plant material was thoroughly extracted with both ethanol–benzene and hot water, but the possibility has not been eliminated that some close physical or chemical association of the administered *p*-hydroxybenzaldehyde distinct from true incorporation into the polymer has taken place (cf. (24)) and that after oxidation a portion of the administered compound was merely recovered unchanged.

The possibility of a $C_6,C_2 + C_1$ type of condensation has been studied by the feeding of mandelic acid labelled in the ring- and 2-carbons. This naturally occurring C_6,C_2 compound was not, as shown in Table I, incorporated into any phenolic aldehyde from wheat lignin to any measurable extent; this is the only instance in our experience when administration of a radioactive compound to a lignifying plant has not resulted in incorporation of detectable amounts of activity. This finding provides no support for the contention of Frank and Marion (25) that a C_6,C_2 fragment can participate in lignin synthesis. These authors base their conclusion on the C^{14}-content of an isolated lignin after administration of hordenine-1-C^{14}, but in view of the very high nitrogen content of their preparation (5.8%) it is probable that the activity was in associated nitrogenous material not forming part of the true lignin polymer.

The effect on the utilization of phenylpropane compounds of the number and location of hydroxyl functions in the 2- and 3-positions of the side chain has been investigated. In Table I the DL-forms of phenyllactic, phenylhydracrylic, and the (dihydroxylated) phenylglyceric acids have been compared to the reference compound, L-phenylalanine, as lignin precursors. From these data, DL-phenyllactic acid is seen to be converted to lignin with an efficiency comparable to that of L-phenylalanine, the former being utilized about 1.5 to 2 times less efficiently. Both the *threo-* and *erythro-*forms of DL-phenylglyceric acid are much less readily used, from about 10 to 25 times

TABLE I

EFFECT OF VARIATIONS IN SIDE CHAIN STRUCTURE ON UTILIZATION OF AROMATIC COMPOUNDS IN LIGNIFICATION BY WHEAT

Compound administered	Specific activity, μc/mM.	Dose, μM./g dry weight	Specific activity of aldehydes from lignin, μc/mM.			Dilution* (Corr. for dry wt. of plant)		
			Vanillin	Syringaldehyde	*p*-Hydroxybenzaldehyde	Vanillin	Syringaldehyde	*p*-Hydroxybenzaldehyde
L-Phenylalanine-R-C^{14}†	32.0	27.7	0.846	1.1	0.535	37.8	29.1	59.7
Mandelic acid-ring, 2-C^{14}	34.6	30.0	0	0	0	∞	∞	∞
p-Hydroxybenzaldehyde-carbonyl-C^{14}	34.5	40.0	0.023	0.030	1.35	2100	1600	36.6
L-Phenylalanine-R-C^{14}†	43.5	19.6	1.31	1.60	1.18	33.2	29.9	36.8
DL-Phenyllactic acid-ring,3-C^{14}	56.0	19.6	0.864	1.26	1.14	71.9	49.2	54.5
L-Phenylalanine-R-C^{14}†	42.5	20.3	0.804	0.885	0.622	52.8	48.0	68.3
DL-Phenylhydracrylic acid-3-C^{14}	94.0	14.2	0.108	0.179	0.396	609	367	166
DL-*threo*-Phenylglyceric acid-3-C^{14}	126	14.5	0.084	0.148	0.061	1100	609	1500
DL-*erythro*-Phenylglyceric acid-3-C^{14}	125	15.4	0.073	0.188	0.060	1300	505	1600

*Observed dilution equals the value in column 2 divided by that in columns 4, 5, or 6.
†Phenylalanine was randomly labelled, and the specific activity is corrected for the loss of two carbon atoms on oxidation (5).

TABLE II

INCORPORATION OF ENANTIOMORPHS OF PHENYLALANINE AND PHENYLLACTIC ACID INTO LIGNIN

Species	Compound administered	Specific activity, μc/mM.	Dose, μM./g dry weight	Specific activity of aldehydes from lignin, μc/mM.			Dilution* (Corr. for dry wt. of plant)		
				Vanillin	Syringaldehyde	*p*-Hydroxybenzaldehyde	Vanillin	Syringaldehyde	*p*-Hydroxybenzaldehyde
Wheat (I)	L-Phenylalanine-3-C^{14}	208	10.3	1.88	2.47	1.65	111	84.2	126
	D-Phenylalanine-3-C^{14}	208	8.23	0.536	0.712	0.260	310	233	640
(II)	L-Phenylalanine†	43.5	17.0	1.15	1.27	0.933	37.8	34.2	46.6
	(−)-Phenyllactic acid‡	56.0	16.1	0.653	0.986	0.908	81.2	52.9	58.5
	(+)-Phenyllactic acid‡	56.0	14.7	0.472	0.730	0.456	104	66.4	106
Spangletop grass	L-Phenylalanine†	43.5	18.9	0.165	0.226	0.214	264	193	203
	(+)-Phenyllactic acid†	56.0	20.8	0.070	0.089	0.248	880	690	250
Sedge	L-Phenylalanine†	43.5	10.3	0.141	0.254	0.221	308	171	197
	(−)-Phenyllactic acid‡	56.0	8.7	0.063	0.092	0.137	750	520	346
	(+)-Phenyllactic acid‡	56.0	10.1	0.106	0.141	0.174	517	389	316
Buckwheat	L-Phenylalanine†	43.5	16.3	1.09	0.835	0.353	39.9	52.1	123
	(−)-Phenyllactic acid‡	56.0	13.7	0.535	0.876	0.121	88.3	53.8	389
	(+)-Phenyllactic acid‡	56.0	14.9	0.009	0.008	0.008	6000	6000	6000
Salvia	L-Phenylalanine†	25.9	20.8	0.459	0.460	0.156	56.5	56.3	166
	(−)-Phenyllactic acid‡	34.6	17.0	0.511	0.658	0.159	55.4	43.0	178
	(+)-Phenyllactic acid‡	36.5	37.8	0.110	0.155	0.153	620	429	435
Maple	L-Phenylalanine†	43.5	20.2	0.485	0.194	—	89.6	224	—
	(−)-Phenyllactic acid‡	56.0	15.6	0.315	0.120	—	137	361	—

*Observed dilution equals the value in column 3 divided by that in columns 4, 5, or 6.
†Phenylalanine was randomly labelled, and the specific activity is corrected for the loss of two carbon atoms on oxidation (5).
‡The phenyllactic acid isomers were labelled in the ring and carbon-3.

more poorly than L-phenylalanine. Such incorporation of the phenylglyceric acids as does occur probably proceeds by dehydration to phenylpyruvic acid:

$$C_6H_5—CHOH—CHOH—COOH \xrightarrow{-H_2O} C_6H_5—CH=COH—COOH \rightleftharpoons C_6H_5—CH_2—CO—COOH.$$

Inasmuch as there is no evidence for the natural occurrence of the phenylglyceric acids in plants, there is no reason to think that these reactions are normally of any importance.

The degree to which the 3-hydroxylated metabolite, DL-phenylhydracrylic acid, is utilized indicates this compound to be intermediate in this respect between phenyllactic and the phenylglyceric acids. While lignin is believed to contain a hydroxy-, a phenoxy-, or an alkoxy- group in the 3-position of the side chain (3), introduction of a hydroxyl at the monomeric stage apparently interferes to some extent with further reactions. This is, of course, consistent with the view that the lignin monomer is a substituted cinnamyl alcohol. The fact that phenylhydracrylic acid is appreciably metabolized suggests that it can be converted to cinnamic acid; by reactions amounting to a dehydration at the 2,3-position the wheat plant can introduce a double bond into the side chain when the 3-carbon bears the hydroxyl function, although less readily than when the 2-carbon is hydroxylated.

Although the reaction sequence by which the prephenic acid – phenylpyruvic acid – phenylalanine system is converted by wheat into the appropriately substituted cinnamyl alcohols remains to a considerable extent hypothetical, the following conversions are consistent with known data:

$$R—CH_2—CO—COOH \rightarrow R—CH_2—CHOH—COOH \rightarrow R—CH=CH—COOH$$
$$\downarrow$$
$$R—CH=CH—CH_2OH.$$

Certainly the results of the experiments reported here give no reason to doubt that the pathway involving phenyllactic acid is the normal one in this species.

The question of stereospecificity has been studied in the case of C_6,C_3 precursors substituted at carbon-2 of the side chain. In Table II D-phenylalanine is compared with L-phenylalanine as a lignin precursor in wheat. D-Amino acid transamination has been confirmed to occur in *Bacillus subtilis* (26). In any case, plants generally seem capable of utilizing a wide variety of phenylpropane compounds, and the moderate incorporation of D-phenylalanine shows that, in this case, stereospecificity is not a very critical factor.

Table II also compares the utilization of the (+)- and (−)- forms of phenyllactic acid-ring,3-C^{14} with that of L-phenylalanine in several species. It shows that in all five species tested (−)-phenyllactic acid was a relatively good precursor, in no case being used more than three times less efficiently than L-phenylalanine in formation of the various aldehyde-yielding residues, and in one case as much as 1.3 times more efficiently. A marked species difference is noted in the utilization of (+)-phenyllactic acid among five species. While it is almost as good as the (−)-form in wheat, and actually better in the sedge, buckwheat, which utilizes the (−)-form well, uses the (+)-form only about

one-hundredth as well as L-phenylalanine. *Salvia* represents an intermediate case, in which the (+)-form of phenyllactic acid is appreciably utilized, but still much more poorly than the (−)-form. These results demonstrate the great variations which can exist among plant species in the proportion of the enzymes involved in the metabolism of enantiomorphs, and suggest that in this case the (−)-metabolizing system is predominant in the dicotyledons.

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References

1. KRATZL, K. and BILLEK, G. Tappi, **40**, 269 (1957).
2. FREUDENBERG, K. Progr. in Chem. Org. Nat. Prods. **11**, 43 (1954).
3. ADLER, E. Tappi, **40**, 294 (1957).
4. BROWN, S. A. and NEISH, A. C. Can. J. Biochem. and Physiol. **33**, 948 (1955).
5. BROWN, S. A. and NEISH, A. C. Can. J. Biochem. and Physiol. **34**, 769 (1956).
6. BROWN, S. A. and NEISH, A. C. Nature, **175**, 688 (1955).
7. EBERHARDT, G. and SCHUBERT, W. J. J. Am. Chem. Soc. **78**, 2835 (1956).
8. BIRCH, A. J. and DONOVAN, F. W. Australian J. Chem. **6**, 360 (1953).
9. WATKIN, J. E., UNDERHILL, E. W., and NEISH, A. C. Can. J. Biochem. and Physiol. **35**, 229 (1957).
10. GATTERMANN, L. Laboratory methods of organic chemistry. Translated from the 24th German edition. Macmillan & Co., Ltd., London. 1952. p. 227.
11. KOO, J., FISH, M. S., WALKER, G. N., and BLAKE, J. Org. Syntheses, **31**, 35 (1951).
12. FITTIG, R. and BINDER, F. Ann. **195**, 131 (1879).
13. LA MER, V. K. and GREENSPAN, J. J. Am. Chem. Soc. **56**, 1492 (1934).
14. BOESEKEN, J. Rec. trav. chim. **41**, 199 (1922).
15. CHUNG, A. and ISRAEL, G. C. J. Chem. Soc. 2667 (1955).
16. RIIBER, C. N. Ber. **48**, 823 (1915).
17. BILLIMORIA, J. D. and COOK, A. H. J. Chem. Soc. 2323 (1949).
18. OVERBY, L. R. and INGERSOLL, A. W. J. Am. Chem. Soc. **73**, 3363 (1951).
19. DAKIN, H. D. and DUDLEY, H. W. J. Biol. Chem. **18**, 29 (1914).
20. BREDERECK, H., VON SCHUH, H., and MARTINI, A. Chem. Ber. **83**, 201 (1950).
21. MORI, Y. Z. physiol. Chem. **122**, 186 (1922).
22. NEUBERG, C. and HIRSCH, J. Biochem. Z. **115**, 282 (1921).
23. HIGUCHI, T., ITO, Y., and KAWAMURA, I. J. Japan. Forestry Soc. **37**, 239 (1955).
24. FREUDENBERG, K. Angew. Chem. **68**, 508 (1956).
25. FRANK, A. W. and MARION, L. Can. J. Chem. **34**, 1641 (1956).
26. THORNE, C. B. *Quoted by* A. Meister *in* Advances in Enzymol. **16**, 185 (1955).

THE USE OF FURFURAL FOR THE DETERMINATION OF ACETONE BODIES IN BIOLOGICAL FLUIDS[1]

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Abstract

A rapid and sensitive method for the determination of acetone plus acetoacetate, and total acetone bodies in blood and urine is described. It is based upon the condensation of acetone and furfural in the presence of a strong base to form difurfurylidene–acetone, which develops a red to violet color in strong acid. A standard Thunberg tube, which is heated and cooled in a water bath, is used as a microrefluxing and a microdistillation unit. The effective range is from 1 to 6 μg of acetone per sample. The average standard deviation, based on recoveries of added material, was 5%. When the method was applied to determinations of blood and urine of several species, the results agreed with those reported by others.

Introduction

The first accurate micromethod for the determination of acetone bodies in biological fluids was introduced by Greenberg and Lester in 1944. Although their method (6, 7), or one of its several modifications (8, 9, 14, 15, 16), remains as the micromethod of choice, it is time consuming, it requires extensive equipment, and it does not lend itself readily to routine analyses. Recently, Bloom (4) has proposed a simple and compact unit which will permit the analysis of many samples at the same time. A Thunberg tube serves as both a microrefluxing and a microdistillation vessel. The lower portion is heated and the hollow stopper is simultaneously cooled in a water bath of simple design. In the original studies with this unit, salicylaldehyde in basic solution was employed as the trapping agent and chromogen in the stopper. However, this reagent does not provide sufficient sensitivity to permit the use of small samples, the blanks are high, and the variations encountered are great (10). As Bahner (2) has comprehensively demonstrated, the variations are due to the reaction between salicylaldehyde and the base. This reaction can be prevented by eliminating light and air from the system. Consequently, in the method outlined by Bahner (1), the equipment and time required are extensive.

The use of furfural as a color reagent for acetone was suggested by Étienne(5). We have found that it serves both as a trapping agent and as a chromogen in the microdistillation unit described by Bloom (4). It permits greater sensitivity than any color reagent yet described. Because only small concentrations are required, the blanks are negligible, the variation is reduced,

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[2]Life Insurance Medical Research Fellow (1954–56), and Lederle Medical Faculty Award (1956–59).

and the reagent is more stable than salicylaldehyde. The method described here is sensitive to 0.5 μg of acetone, and the effective range is from 1–6 μg with an error of about 5%. β-Hydroxybutyrate was recovered quantitatively.

Methods

Principle

Acetoacetate is decomposed to acetone by heat and acid and, together with the preformed acetone, is distilled into the hollow stopper of a Thunberg tube. Here the acetone is trapped by furfural in alkaline solution. On the addition of strong acid to the difurfurylidene–acetone in the stopper, a red to violet color develops instantly. Total acetone bodies are determined in the same way, except that potassium dichromate is added for the oxidation of β-hydroxybutyrate.

Reagents

Furfural, 0.1% (v/v), distilled; 5 *N* potassium (or sodium) hydroxide; 20 *N* sulphuric acid; 4 *N* sulphuric acid; 0.75% (w/v) potassium dichromate; 0.5 *N* sodium hydroxide; and 10% (w/v) zinc sulphate. Ten milliliters of the zinc sulphate must be equivalent to 10.8–11.2 ml of the sodium hydroxide with phenolphthalein used as the indicator (13).

Apparatus

Standard Thunberg tubes and the water bath described by Bloom (4) are used. Recently, we have learned that the dimensions and the shape of the hollow stopper of the Thunberg tube are of some importance. Unless the stopper is shaped to provide minimal constriction, the distillation of acetone will not be complete. The tube and stopper shown in Fig. 1 has produced satisfactory results. In general, the shape of the stopper resembles that of a boot, and the elongated area permits the greater portion of it to be immersed in the cooling troughs of the water bath.

Preparation of Standards and Samples

As described, this method is accurate for concentrations of acetone from 1–6 μg per ml. A stock standard of acetone containing about 0.4 mg/ml is prepared from redistilled acetone and the exact concentration is determined by Messinger's iodometric titration (10). This standard is stable for 30–60 days. Working standards are prepared daily from this stock.

Protein-free filtrates of whole blood are prepared in screw-capped centrifuge tubes. To blood laked in the desired volume of water, the sodium hydroxide and the zinc sulphate, one volume of each per volume of blood, are added in that order, and the solution is thoroughly stirred after each addition. Samples of 0.5 ml are required for blood from normal, non-fasting subjects, but samples of 0.05 ml suffice for blood from fasting or pathological subjects.

Filtrates of urine are prepared in the same way. If the concentration of acetone plus acetoacetate only is desired, samples of whole blood or urine may be added directly to 1 ml of 4 *N* sulphuric acid in the Thunberg tube.

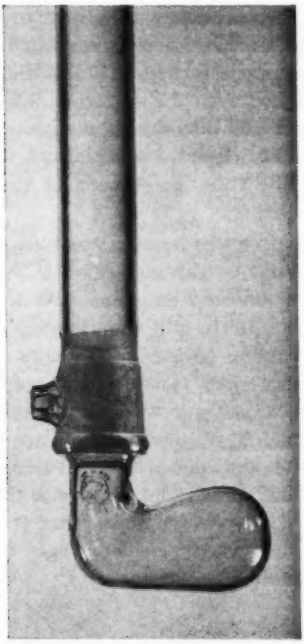

FIG. 1. Thunberg tube and stopper. The tube is 17 mm (O.D.)×140 mm long with a standard 14×20 tapered joint. The hollow stopper, approximately 18×40 mm, has a total capacity of 6–7 ml.

Determination of Total Acetone Bodies

The hollow stoppers are prepared first with a thin layer of silicone stopcock grease* on the ground-glass joint. With the aid of a 0.1-ml pipette (or a calibrated syringe) equipped with an 18-gauge hypodermic needle, 0.1 ml of 5 *N* potassium hydroxide is deposited as a single drop in the "heel" of each stopper. One milliliter of 4 *N* sulphuric acid and then 1 ml of filtrate or standard solution are transferred to the main (Thunberg) tubes. The tubes are corked temporarily. A reagent blank containing distilled water in place of filtrate is prepared in the same way. The last addition made is furfural; 0.5 ml is transferred to the hollow stoppers by a pipette with a thin, elongated tip. It is essential that all of the furfural be placed in the "heel" of the stopper. The stoppers and the tubes are joined, firmly sealed with a strip of adhesive tape, and placed in the water bath. The temperature of the hot water must be greater than 95° C. A temperature of tap water as high as 26° C in the cooling troughs will still permit complete distillation.

The tubes are heated for 15–20 minutes, during which time all of the acetoacetate is decomposed to acetone. At this time potassium dichromate is added to the tubes for the oxidation of β-hydroxybutyrate. If it is added

*Dow Corning high vacuum grease. Soaking the tubes and the stoppers for 30 minutes in a warm solution of 5–10% potassium hydroxide removes all of the grease.

earlier a fraction of the acetoacetate will be destroyed. All of the tubes are removed from the bath and rapidly cooled to room temperature. The adhesive strip is removed, and the tube is turned, keeping the stopper stationary, until the hole in the tube is aligned with the sidearm. One milliliter of 0.75% potassium dichromate is delivered into the tube with a hypodermic syringe and needle. The tube is quickly closed, the adhesive strip is replaced, and the solution is mixed before the tube is returned to the bath for an additional heating period of 60 minutes.

At 15-minute intervals after this heating has begun, the tubes are removed from the bath and swirled so that the surface of the furfural is renewed. This procedure is necessary for efficient trapping. At the end of the period the tubes are removed from the bath, and the stoppers are removed from all of the tubes at once. No cooling period is necessary. The small amounts of water which have condensed just inside of the stoppers in the area of the ground glass are carefully wiped out with hard paper toweling. Two milliliters (or 3 ml see Fig. 4A) of 20 *N* sulphuric acid are added to each stopper in the same order in which the additions of furfural were made, and the solutions are thoroughly mixed. The red to violet solution is then poured into a cuvette and the density is measured in a spectrophotometer at a wavelength of 530 mμ. All of the determinations reported here were read in 10–12 mm tubes in a Coleman Jr. spectrophotometer.

Determination of Acetone plus Acetoacetate

This fraction is determined in aliquots of the same filtrates used for total acetone bodies. Instead of potassium dichromate, 1 ml of distilled water is added to each tube. This extra volume of liquid is necessary to eliminate any possibility of unequal quantities of water condensing in the stoppers.

Results

Choice of Reagents

The justification for the use of furfural as a chromogen is given in Fig. 2. The reaction between the base and furfural yields increasing color with time, and the rate of the development of the color is increased with higher concentrations of base. However, when only a limited supply of air is present, as occurs in the closed tube during distillation, and when a low concentration of base is used, the color developed in the blanks is minimal. Indeed, between the first and second hour (Fig. 2), no color change occurs at all. As described above, the entire procedure is completed within 2 hours. Any variations induced by this reaction can be estimated by using distilled water blanks as the first and last tubes in a series.

Optimal sensitivity was achieved with low concentrations of furfural (0.1%). Minor gains in sensitivity were obtained with higher concentrations, but only at the cost of large increases in the color of the blanks (Fig. 3A). At this concentration of furfural, the presence of 5 *N* base is required for optimal color

development (Fig. 3B). It was with this concentration of base that the reaction between base and furfural was found to be minimal (Fig. 2). A 0.1% solution of furfural has the additional advantage of being stable at room temperature and in the light.

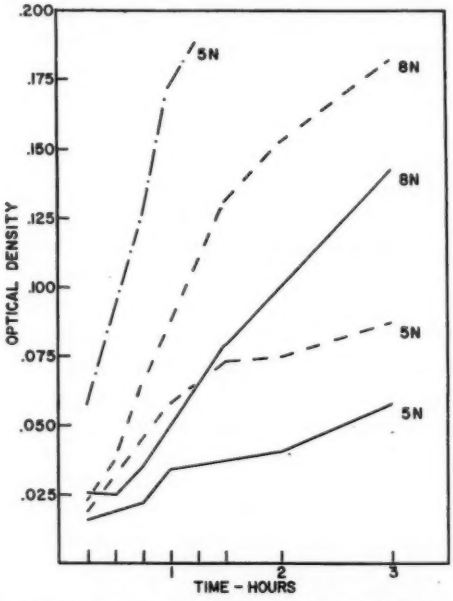

FIG. 2. Changes in color density of furfural and potassium hydroxide with time. Mixtures of 0.5 ml of 0.1% furfural and 0.1 ml of 5 or 8 *N* potassium hydroxide were allowed to stand in open test tubes (dashed lines), or corked tubes (solid lines), which were cooled in tap water. At the indicated time intervals, 3 ml of 20 *N* sulphuric acid were added, and the solutions were read at a wavelength of 530 mμ. The dashed and dotted line represents the samples containing 5 *N* potassium hydroxide in the corked tubes but read at 410 mμ.

As shown in Fig. 4A, the sensitivity of the reaction may be reduced as required by increasing the volume of sulphuric acid used to develop the color. Volumes less than 2 ml are inadequate since the colored product formed rapidly fades in the light. Roughly 30 milliequivalents of acid must be present in excess to yield a stable product. Acid concentrations above 20 *N* serve only to increase the color of the blank (Fig. 4B). With the optimal concentrations of acid, base, and furfural, the final colored product fades slowly, at the rate of about five per cent per hour. With these concentrations of base and furfural, the volumes may be altered by 50% from those recommended above without seriously changing the final color development.

Interference

By this method final concentrations of potassium dichromate between 0.07 and 0.25% are optimal for the oxidation of β-hydroxybutyrate. It is well known that the presence of glucose or lactic acid in high concentrations will

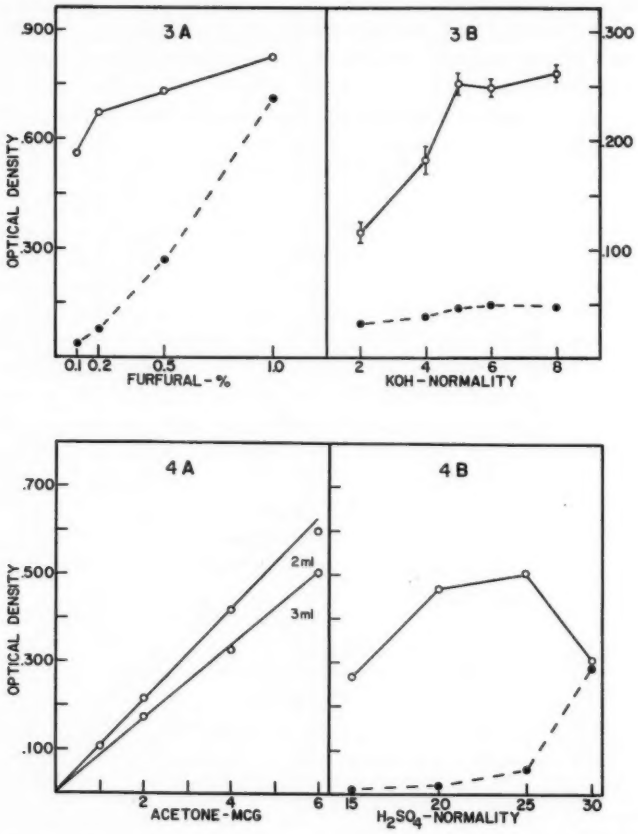

FIG. 3. Comparison of concentrations of furfural (A) and potassium hydroxide (B) on color development.

A. Each point represents 2–10 determinations of 8 μg of acetone (solid lines and open circles). The distilled water blanks (dashed lines and closed circles) were read against distilled water. The procedure as described was followed except that 8 *N* (0.05 ml) potassium hydroxide was used.

B. Each point represents 8–10 determinations of 2 μg of acetone (solid lines and open circles). The distilled water blanks (dashed lines and closed circles) were read against distilled water. The vertical bars are the standard deviations of the means. The procedure as described was followed.

FIG. 4. Comparison of volumes (A) and concentrations (B) of sulphuric acid on color development.

A. Each point represents 9–10 determinations of 1–6 μg of acetone with either 2 or 3 ml of sulphuric acid. The procedure as described was followed.

B. Each point represents 2 determinations of 8 μg of acetone (solid lines and open circles). Distilled water blanks (dashed lines and closed circles) were read against distilled water. One milliliter of acetone or distilled water was mixed with 1 ml of 0.1% furfural and 1 ml of 60% potassium hydroxide. After 30 minutes 10 ml of sulphuric acid were added, and the optical density was read at a wavelength of 530 mμ.

prevent this reaction by reducing the dichromate. Quantities of glucose or lactic acid up to 50 μg added to standard solutions did not interfere, but quantities from 0.1 to 1 mg did. Thus, concentrations of glucose and lactic acid less than 165 mg per 100 ml of blood (1 to 33 dilution) are within safe limits.

Within these concentrations of dichromate, citric acid is oxidized to acetone, but in only 20–25% of the theoretical yield. The presence of 15 μg of citric acid would be necessary to yield the equivalent of 1 μg of acetone (46 mg per 100 ml of blood). With the protein precipitants suggested above citric acid is completely removed from solution.

As reported for salicylaldehyde (14), acetaldehyde condenses with furfural to yield a yellow- to brown-colored product in the presence of acid. Ethanol is partially oxidized to acetaldehyde by the acid dichromate. The production of a colored compound equivalent to 0.1 μg of acetone would require 2.3 μg of acetaldehyde and 11.4 μg of ethanol, or 7.3 and 36.5 mg per 100 ml of blood, respectively. Neither urea nor cholesterol showed any interference.

Standards and Variations

The relationship between concentration and optical densities of 1–6 μg quantities of acetone is presented in Fig. 4A. The mean values were calculated from determinations made over a 10-day period, and the standard deviations ranged from 2.5 to 7.5%. As an estimation of the precision of the method, the differences between duplicate determinations were compared for 1-, 2-, and 4-μg quantities of acetone. A series of 25 duplicate determinations for each of these amounts yielded standard deviations of 5.2, 4.9, and 3.7% respectively.

Recovery Studies

Recoveries of acetone and β-hydroxybutyrate from aqueous solutions, plasma, and whole blood are given in Table I. The quantitative recovery of β-hydroxybutyrate (calculated throughout in acetone equivalents) both from aqueous solutions containing acetone and from plasma was unexpected since 80% yields of acetone from β-hydroxybutyrate have become almost an accepted error. On the other hand, pure solutions of β-hydroxybutyrate (sodium salt) yielded acetone equivalents of only 86% at the maximum. In these studies, final concentrations of potassium dichromate from 0.07–0.25% in 1.3–2.0 *N* sulphuric acid were employed. Final concentrations in this range have been recommended in almost all of the previous methods.

Even though the complete recoveries of β-hydroxybutyrate from aqueous solutions containing acetone were obtained independently by three people in two laboratories, it was not impossible that the apparent complete recoveries were due to the presence of more than the calculated amount of acetone. To eliminate this possibility, the amount of β-hydroxybutyrate was calculated by subtracting the amount of acetone found without oxidation from the total amount found. The recovery ratio is then solely in terms of β-hydroxybutyrate (Table I, last column). This method of calculation would obviously double the error. If, on the other hand, the recovery ratios of the aqueous

mixtures are calculated in terms of the total amount found (acetone plus β-hydroxybutyrate), then mean percentages of 98±2 (standard deviation) and 99±1 were found for the mixtures of 1.0 and 1.0, and 2.0 and 2.0 μg of acetone and β-hydroxybutyrate, respectively. Mixtures of 0.5 and 0.5, 0.8, and 0.5 μg of acetone and β-hydroxybutyrate, respectively, yielded total recoveries of 103±4 and 99±9%. These are the means and standard deviations of three and four determinations in duplicate.

TABLE I

RECOVERY STUDIES WITH ACETONE AND β-HYDROXYBUTYRATE

Substance added, μg		No. of determinations	Av. initial level, μg/tube	Substance recovered*	
Acetone	β-Hydroxybutyrate			Acetone	β-Hydroxybutyrate
		Recovery from aqueous mixtures			
1.0	1.0	5	0.00	107±6†	91±5
2.0	2.0	3	0.00	97±1	101±3
		Recovery from plasma			
0.8		3	0.77	103±6	
1.0		3	0.76	96±4	
1.6		3	0.77	103±5	
3.2		3	0.77	101±2	
	0.5	4	1.61		95±7
	1.0	10	1.70		100±3
	1.5	4	1.88		99±6
	2.0	6	1.69		100±5
	4.0	6	1.69		99±5
		Recovery from whole blood			
1.0		9	0.81	99±7	

*Calculated from (amount found/amount expected) × 100 where the amount expected was the sum of the initial level and the amount added. All calculations were in terms of acetone equivalents.
†Standard deviation of the mean.

In the recovery studies with plasma, samples of whole blood were laked in solutions of acetone or β-hydroxybutyrate, and aliquots of the protein-free filtrates were analyzed in duplicate. For amounts of 1–4 μg of β-hydroxybutyrate, the standard deviations of the recovery ratios ranged from 3 to 6% (Table I). This error is considerably lower than that found by Bloom (4) using salicylaldehyde as a chromogen. In the range of 2 to 4 μg of β-hydroxybutyrate, his standard deviation for 10 determinations was 8.7%.

The standard deviations of the recovery ratios of acetone and β-hydroxybutyrate from plasma and whole blood ranged from 2 to 7% for additions of 0.5 to 4.0 μg of material (Table I). The composite standard deviation for these 51 determinations was 4.8%.

Application to Biological Fluids

The concentrations of acetone bodies in blood and urine of several species of animals are given in Table II. These results are in the range of those reported by others (3, 6, 11).

TABLE II

THE LEVELS OF ACETONE BODIES IN BLOOD AND URINE OF FOUR SPECIES

Species	Sex	No. of determinations	Fed or* fasted	Fluid	Acetone plus acetoacetate,† mg per 100 ml	Total acetone† bodies
Man	Female	2	Fed	Blood	0.34, 0.52	0.76, 1.10
		2	Fasted		0.63, 0.72	1.45, 1.06
		2	Fed	Urine‡	0.24, 0.42	0.79, 0.55
		2	Fasted		0.51, 0.49	1.44, 1.49
	Male	2	Fed	Blood	0.22, 0.34	0.53, 0.78
		2	Fasted		1.78, 1.11	3.71. 2.90
		2	Fed	Urine‡	0.24, 0.42	0.79, 0.55
Dog	Male	3	Fed	Blood	0.30, 0.30 0.29	0.47, 0.50 0.85
Rat	Male	4	Fasted	Blood	2.44±0.22§	5.03±0.61
Mouse	Male	5	Fasted	Blood	3.84±0.37	11.08±0.36

*All fasting periods were from 24 to 30 hours.
†All calculations were in terms of acetone.
‡Twenty-four hour specimens.
§Standard deviation of the mean.

Discussion

The efficiency, reproducibility, and sensitivity of this method quite clearly depend, in part, upon the reliability of the Thunberg tube as a microdistillation and microrefluxing unit and, in part, on the value of furfural as a chromogen and trapping agent. The efficiency of the Thunberg tube rests on the fact that acetone is distilled directly into the trapping agent without surplus volumes of water which serve only to dilute the distillate. Most of the water which does condense within the stopper may be removed. Because no two hollow stoppers have the same dimensions or shapes, the amount of water condensing in them might be expected to vary. Determinations by weight of the water of condensation revealed a variation of less than 2% from stopper to stopper. With the color test described here, it was found that from 2 to 5% of the acetone was not distilled into the stopper.

Despite the report by Shipley and Long (12) that small amounts of acetone are lost when pure solutions are heated in sealed glass vessels, distillation appeals as the method of choice for the removal of acetone from biological materials. It reduces the possible number of interfering compounds and the dangers involved in several manipulations of the sample which accompany precipitation and extraction procedures.

The use of furfural has eliminated many of the difficulties encountered with salicylaldehyde. The most serious objection to furfural is the reaction with base which yields increasingly darker solutions with time. The rate of these reactions may be increased with higher concentrations of base (Fig. 2). Bahner (2) has demonstrated that the increase in color development which occurs between salicylaldehyde and base may be eliminated by the complete

exclusion of both light and air from the vessels containing the reagents. With furfural, it has been found that when a continuous supply of air is excluded from the reaction, as occurs during the present distillation process, the color change is minimal (Fig. 2); indeed, between 1 and 2 hours little or no change occurs when potassium hydroxide concentrations of 5 *N* are employed. The reagent is not ideal, but if the precautions suggested here are taken, low variations may be achieved.

Acknowledgment

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References

1. BAHNER, F. Biochem. Z. **323**, 318 (1952).
2. BAHNER, F. Biochem. Z. **323**, 327 (1952).
3. BAHNER, F. Acta Endocrinol. **13**, 213 (1953).
4. BLOOM, W. L. J. Lab. Clin. Med. **51**, 824 (1958).
5. ÉTIENNE, H. Ind. chim. belge, **17**, 455 (1952). *Cited in* Chem. Abstr. **17**, 455 (1953).
6. GREENBERG, L. A. and LESTER, D. J. Biol. Chem. **154**, 177 (1944).
7. LESTER, D. and GREENBERG, L. A. J. Biol. Chem. **174**, 903 (1948).
8. MAYES, P. A. and ROBSON, W. Biochem. J. **67**, 11 (1957).
9. MICHAELS, G. D., MARGEN, S., LIEBERT, G., and KINSELL, L. W. J. Clin. Invest. **30**, 1483 (1951).
10. PETERS, J. P. and VAN SLYKE, D. D. Quantitative clinical chemistry. Vol. II. The Williams and Wilkins Co., Baltimore, Md. 1932. pp. 623–646.
11. ROSENTHAL, S. M. J. Biol. Chem. **179**, 1235 (1949).
12. SHIPLEY, R. A. and LONG, C. N. H. Biochem. J. **32**, 2242 (1938).
13. SOMOGYI, M. J. Biol. Chem. **86**, 655 (1930).
14. THIN, C. A. and ROBERTSON, A. Biochem. J. **51**, 218 (1952).
15. WEINSTEIN, H. R. and FOÀ, P. P. Am. J. Physiol. **163**, 759 (1950).
16. WERK, E. E., JR., MCPHERSON, H. T., HAMRICK, L. W., JR., MYERS, J. D., and ENGEL, F. L. J. Clin. Invest. **34**, 1256 (1955).

THE METABOLISM OF THE ERYTHROCYTE

XV. ADENYLATE KINASE OF THE ERYTHROCYTE[1]

SHELBY KASHKET[2] AND ORVILLE F. DENSTEDT

Abstract

Adenylate kinase has been found in the membrane and the cytoplasm of rabbit and human erythrocytes. ADP, when added to a suspension of washed erythrocytes, causes an increase in the rate of glucose utilization by the cells. The ADP evidently can enter the cell membrane where it is converted by the adenylate kinase to equimolar quantities of ATP and AMP. The ATP can pass either into the interior of the cell or into the external medium. Added ATP or AMP apparently cannot pass through the erythrocyte membrane. The role of adenylate kinase in these reactions, and its location within the erythrocyte membrane, are discussed.

Introduction

The enzyme, adenylate kinase, was discovered by Colowick and Kalckar (1, 2) in skeletal muscle. It catalyzes the following interconversion which Kalckar (2) has referred to as a "phosphate dismutation":

$$2ADP \rightleftharpoons ATP + AMP.*$$

The enzyme occurs also in other tissues (1, 2, 5, 6, 7) and has been referred to as adenosine diphosphoric acid phosphomutase (3), myokinase (1, 2), and adenylate kinase (4). In this paper evidence is given for its presence in the cytoplasm and the stroma of the erythrocyte.

Experimental

1. Reagents

Glycylglycine, adenosine mono-, di-, and tri-phosphates, inosine monophosphate, and yeast hexokinase were obtained from Nutritional Biochemicals Corporation. Chromatographic analysis of the nucleotides revealed that the ATP contained a trace of ADP, and the ADP, a trace of AMP. The AMP and IMP were found to be pure.

2. Methods

Blood from rabbits was drawn from the ear vein into centrifuge tubes containing crystalline heparin. Blood from human subjects was collected into ACD medium or mixed with heparin. In each case the erythrocytes were washed three or four times with isotonic (0.154 *M*) KCl and each time the

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*The following abbreviations are used in the text: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate or adenylic acid; IMP, inosine monophosphate or inosinic acid; SFH, stroma-free hemolyzate; ACD, acidified citrate–dextrose preservative medium. (Dextrose, 1.47 g; trisodium citrate, 1.32 g; citric acid, 0.44 g in 100 ml of solution.)

overlying layer of white cells was removed by careful aspiration. The red cells finally were suspended in sufficient isotonic KCl to give a volume equal to that of the original blood sample.

For the preparation of hemolyzates a quantity of the washed cell suspension was diluted with two volumes of cold distilled water. The mixture was placed in the refrigerator for 20–30 minutes after which time the salt concentration was restored to isotonicity by the addition of 0.2 volumes of 1.54 *M* KCl. The whole hemolyzate was centrifuged at $600 \times g$ for 3 minutes and the clear stroma-free supernatant (SFH) was carefully removed. The stroma residue was washed several times with distilled water and finally with isotonic KCl. The pink-colored residue was finally suspended in sufficient isotonic KCl to make the volume equal to that of the original whole hemolyzate.

The enzyme preparations from human blood were incubated at 37° C in media with the following ingredients: 0.013 *M* phosphate buffer; 0.027 *M* glycylglycine buffer, pH 8; 0.005 *M* $MgCl_2$; 0.0022 *M* glucose; 0.005 *M* adenosine mono-, di-, or tri-phosphate, or equimolar mixtures of these nucleotides; and sufficient isotonic KCl to make the final volume up to 1.5 ml. The final pH was 7.8. All the ingredients were prepared in isotonic KCl. The solutions of the nucleotides were neutralized with $KHCO_3$ immediately before they were added to the medium. In certain experiments yeast hexokinase (0.06 ml of a stock solution containing 100 mg of the enzyme per ml) was added to the incubation medium. The amount of glucose utilized during the experimental period was estimated according to the method of Nelson (8).

In experiments which required the identification and estimation of the nucleotides glucose was omitted from the medium. At the end of the incubation period 3.5 ml of cold 2% perchloric acid was added to the medium to stop the reaction and to precipitate the proteins. After centrifugation 0.10 ml of the supernatant from each specimen was applied to the paper strips in a Spinco electrophoresis apparatus (Durrum type with 0.05 *M* citrate buffer, pH 4.8). In some of the runs the electrophoretic separation was continued for 16 hours at 100 volts, and in others, for 4 hours at 200 volts. An effort to recover quantitatively the individual nucleotides from the paper strips by elution with water proved unsuccessful. Instead, therefore, photographic prints of the dried paper strips were made under ultraviolet light with a Corning U. V. filter No. 9863.

Results

1. Studies with Cell-free Preparations

(*1*) *Influence of added nucleotides on glucose utilization.*—A comparison of the influence of various nucleotides and mixtures of these nucleotides on the glucose utilization of the stroma-free hemolyzate and of erythrocytes is given in Table I. The presence of adenylate kinase in the erythrocyte was first observed with the SFH from rabbit cells. In the presence of ADP the

SFH from the human red cell preparations used in the present study was found to utilize glucose in an amount equivalent to about 10% of the added nucleotide.

TABLE I

ADENYLATE KINASE ACTIVITY OF THE HUMAN ERYTHROCYTE

Nucleotide added	Glucose utilization, μg/hr			
	Intact erythrocytes		Stroma-free hemolyzate	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
–	75	142	–	–
ATP	38	127	73	80
ADP	124	250	52	62
AMP	19	78	–	0
ATP + ADP	–	213	48	43
ATP + AMP	–	108	–	67
ADP + AMP	–	209	–	35

NOTE: 0.5–0.8 ml enzyme preparation incubated with 0.013 *M* phosphate buffer and 0.027 *M* glycylglycine buffer, pH 8; 0.005 *M* $MgCl_2$; 0.0022 *M* glucose; 0.005 *M* of each nucleotide; 0.154 *M* KCl to make the final volume up to 1.5 ml. Incubation temperature, 37° C.

(*2*) *Influence of added yeast hexokinase.*—In order to confirm that ATP was formed from the ADP added to the SFH preparations, purified yeast hexokinase was added to the external medium and the rate of glucose utilization was measured. Yeast hexokinase was used because it has a specific requirement for ATP (1). It is evident from the results presented in Table II that the glucose utilization was greatly increased in all three preparations in the presence of the yeast enzyme. The adenylate kinase was present, therefore, in both the stroma and the SFH.

TABLE II

ADENYLATE KINASE ACTIVITY OF THE ERYTHROCYTE AND OF THE SOLUBLE AND INSOLUBLE FRACTIONS

	Yeast hexokinase	Glucose utilization, μg/30 min		
		Intact erythrocytes	Stroma-free hemolyzate	Washed stroma
Enzyme	–	29	31	0
–	+	37	23	37
Enzyme	+	482	675	207
Enzyme heated 15 min	+	132	493	49
Enzyme heated 30 min	+	58	25	–
Enzyme heated 15 min	–	–	0	5

NOTE: 0.5 ml SFH, or 0.1 ml intact erythrocytes, or 0.4 ml washed stroma incubated with 0.005 *M* ADP as in Table I; 0.06 ml yeast hexokinase was added where indicated. The preparations, where indicated, were heated at 55–60° C for 15 or 30 minutes respectively, before being added to the incubation media.

(*3*) *Analysis of nucleotides.*—The presence of an adenylate kinase type of phosphorylating mechanism in the blood preparations was confirmed further by chromatographic analysis of the nucleotides. Portions of the deproteinized sample from an incubation mixture, which contained SFH and ADP but no glucose, were subjected to paper electrophoresis. The presence of bands of ATP, ADP, and AMP on the paper strips was clearly demonstrated as indicated in Table III and shown in the 60-minute chromatograms in Fig. 1. A fourth band, namely that of IMP, also was identified. This nucleotide is formed on deamination of AMP by the adenylic acid deaminase in the erythrocyte (9). Frequently, the deaminase of the SFH preparations was so active that all of the AMP was converted to IMP. This circumstance made impractical the measurement of the equilibrium constant for the adenylate kinase system. When ATP and AMP together were incubated with the SFH the formation of ADP was demonstrated by the appearance of the band on the chromatogram.

2. Studies with Intact Erythrocytes

The presence of an adenylate kinase in the membrane of the red cell also was established. From the data presented in Table I it is evident that the addition of ADP to the system increased the rate of glucose utilization by the intact human erythrocyte by 100–150%. The addition of ATP or AMP, on the other hand, actually caused a decrease in the glucose utilization to less than the endogenous rate. Furthermore, when ATP or AMP was added along with ADP in equimolar concentration the stimulatory action of the latter was much reduced. The addition of AMP along with ATP similarly decreased the rate of glucose utilization to less than that obtained with ATP alone.

TABLE III

INTERCONVERSION OF NUCLEOTIDES BY THE ADENYLATE KINASE OF THE HUMAN ERYTHROCYTE

Enzyme preparation	Nucleotide added	Incubation time, min	Nucleotide bands found			
			ATP	ADP	IMP	AMP
SFH	ADP	0	−	++	−	+
		60	++	+	++	(+)
Hemolyzate	ADP	0	−	++	−	(+)
		60	++	+	++	(+)
SFH	ATP + AMP	0	++	−	−	++
		60	++	++	++	−
Erythrocytes	ADP	0	(+)	++	−	(+)
		60	+	+	(+)	+
"	ATP + AMP	0	++	(+)	−	++
		60	++	++	(+)	++
"	ATP	0	++	(+)	−	−
		60	++	(+)	−	−
"	AMP	0	−	−	−	++
		60	−	−	−	++

NOTE: 0.5 ml enzyme preparation incubated as specified in Table I, but without added glucose. The paper strips on which the nucleotides had been separated by electrophoresis were photographed under ultraviolet light. + indicates presence: (+) trace; − absence.

In the course of the experiments it was found that the concentration of inorganic phosphate remained unchanged, and that neither 0.005 *M* arsenate nor 0.0017 *M* iodoacetate caused any inhibition of ATP formation. Inhibition in the presence of either of these poisons would have occurred if an enzyme such as triosephosphate dehydrogenase (10), instead of adenylate kinase, was involved in the reaction.

When ADP was added to the suspension of washed human erythrocytes along with yeast hexokinase the rate of glucose utilization, as indicated in Table II, was increased above that obtained without the yeast enzyme.

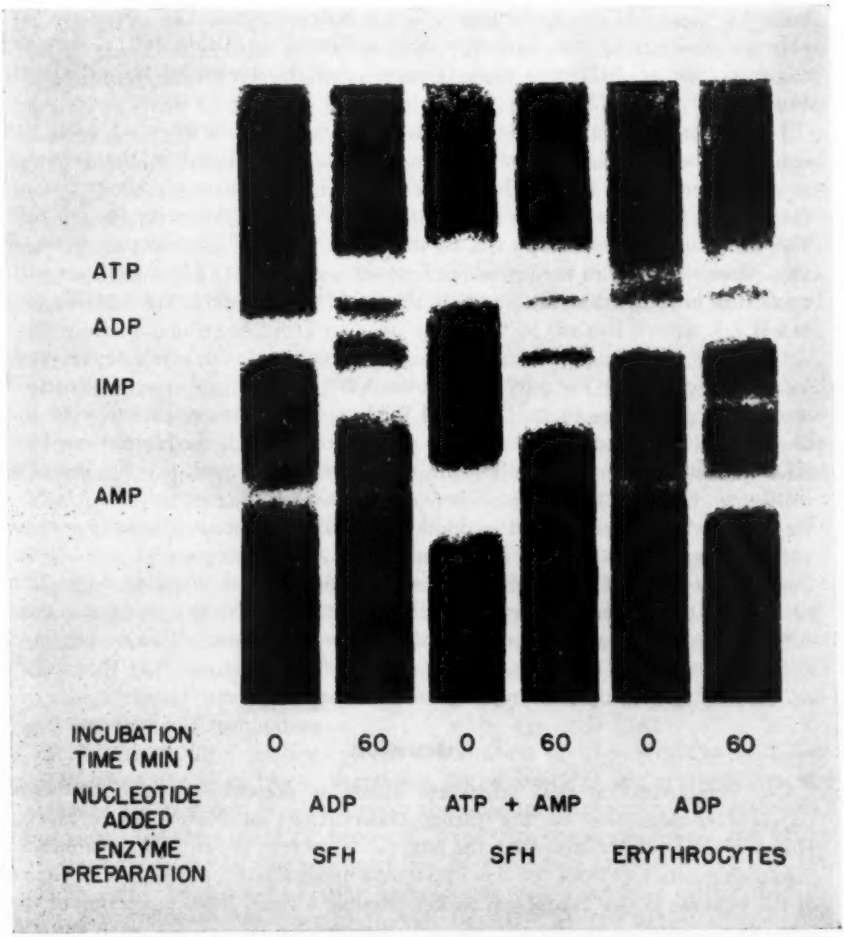

FIG. 1. Chromatographic evidence of the interconversion of added nucleotides by the adenylate kinase of the human erythrocyte.

Evidently some of the ATP, formed at the red cell membrane by phosphorylation of the added ADP, passed into the external medium where it served as a substrate for the yeast hexokinase. We had established that the yeast hexokinase does not enter the erythrocyte nor is it adsorbed onto the surface of the cell. The addition of AMP to the system was found to inhibit the formation of ATP from ADP. The results with preparations of washed stroma were essentially the same as with the intact cells.

The adenylate kinase of the red cell membrane and of the SFH was found to be relatively stable when heated to 60° C in a neutral medium (see Table II).

On incubation of the intact erythrocytes with ADP in the absence of glucose, both ATP and AMP were formed. This was demonstrated by paper electrophoretic analysis of the medium, as is indicated in Table III. Likewise, the formation of ADP was demonstrated after incubation of the cells with added ATP and AMP.

It is of interest that AMP did not undergo deamination when added to the suspension of red cells whereas it was rapidly deaminated in the presence of SFH (see Table III). This confirms the observations of Albaum *et al.* (11) and of Hevesy and Aten (12) that added AMP cannot enter the red cell. The data in Table III indicate, furthermore, that ATP undergoes little, if any, phosphorolysis in the presence of erythrocytes (13). These findings with washed or unwashed erythrocytes in media of pH 7.8 were reproducible also at pH 7.3.

Of special significance is the observation that when the erythrocytes were incubated with ADP, or ATP along with AMP, the newly formed nucleotides were soon detectable in the external medium. This is consistent with the observation indicated in Table II that some of the ATP formed from the added ADP passed into the red cells while the remainder passed into the external medium. On the other hand, when an increase in the intracellular concentration of ATP was induced by preincubation of the red cells with inosine (14), subsequent incubation of the cells with added glucose and yeast hexokinase, but without the addition of nucleotide, yielded no phosphorylation of glucose by the yeast enzyme in the medium. Evidently no transfer of ATP occurs from the cell interior to the external medium. This finding is in agreement with the converse observation of Szekely *et al.* (13) that ATP, added to the external medium, does not enter the erythrocyte.

Discussion

Our demonstration that adenylate kinase is present in the erythrocyte provides confirmation of the earlier observations of Kotel'nikova (3, 5). However, the observation that the enzyme is present in both the soluble and insoluble fractions of the cell has important implications. First, the presence of the enzyme in the cytoplasm makes possible a rapid interconversion of the adenine nucleotides in the cell; second, the presence of the kinase in the cell membrane offers an explanation for the observation that added ADP can give rise to ATP within the cell.

There appears to be some disagreement among workers as to the concentration of the adenine nucleotides in the erythrocyte. Gourley (15) was able to demonstrate the presence of only ATP and AMP in the normal red cell. Prankerd and Altman (16) and Bartlett *et al.* (17) found ATP, AMP, and ADP. The latter observation is consistent with the presence of adenylate kinase in the red cell since it is unlikely that all of the cellular ADP would be converted to ATP by the enzyme. In the present study the interference of the adenylic acid deaminase made it impossible to determine the equilibrium constant of the adenylate kinase. Kalckar (2) found, by using the adenylate kinase from muscle, that about 60% of the added ADP was converted to ATP and AMP at equilibrium. Thus, if the behavior of the adenylate kinase of the erythrocyte is similar to that of muscle, it is to be expected that all three nucleotides will be present and interconvertible.

A characteristic of the adenylate kinase from various sources is its relatively high stability to heat. The kinase of the erythrocyte is much more stable to heating at 60° C in a neutral medium than is that from rat liver (7). Kotel'nikova (5) found that the enzyme from skeletal muscle is not affected by heating in solution at 100° C whereas both the liver and erythrocyte enzymes are rapidly destroyed at this temperature. It is of interest that the adenylate kinase from the 'soluble' fraction of rat liver is stable to heating at 100° C in an acid medium (18) whereas the enzyme from liver mitochondria is rapidly inactivated under these conditions (7). The kinase of the SFH of the red cell compares, in its stability to heat, with that of the cell stroma.

It is probable that the adenylate kinase in the red cell membrane is distributed on, or just beneath, the external surface. This inference is based on the observation that neither ATP nor AMP can pass through the membrane. Furthermore, ATP, added to the medium, does not stimulate the glucose uptake of the cells, nor does added AMP undergo deamination. Added ADP, on the other hand, can enter the external part of the membrane where it undergoes dismutation to ATP and AMP. These products then can pass freely into or out of the cell. Apparently ADP can pass in only one direction through the membrane, namely from the outside inwards, otherwise the cell would rapidly be depleted of its supply of endogenous nucleotides. Siekevitz and Potter (19) have postulated a similar distribution of adenylate kinase in the membrane of rat liver mitochondria. The enzyme evidently can regulate, in a specific manner, the passage of adenine nucleotides into and out of the mitochondria.

Relevant to these findings is the observation of Sventsitskaya (20) that ATP, when added to blood, maintains the viability of the erythrocytes and prolongs the period of preservation in the cells during storage. Sventsitskaya has suggested that the ATP becomes "fixed" by the erythrocytes and brings about an increase in the concentration of the labile phosphate constituents of the cell. In the light of our findings one would surmise rather that the added ATP first undergoes hydrolysis to ADP by the action of the phosphatases of the plasma (21), and that the ADP is then transferred into the erythrocyte by the adenylate kinase mechanism.

References

1. COLOWICK, S. P. and KALCKAR, H. M. J. Biol. Chem. **148**, 117 (1943).
2. KALCKAR, H. M. J. Biol. Chem. **148**, 127 (1943).
3. KOTEL'NIKOVA, A. V. Chem. Abstr. **42**, 6388*c* (1948).
4. COLOWICK, S. P. *In* The enzymes. *Edited by* J. B. Sumner and K. Myrbäck. Academic Press, Inc., New York. 1951. pp. 114–150.
5. KOTEL'NIKOVA, A. V. Chem. Abstr. **43**, 6263*h* (1949).
6. BAKULIS, S. S. and LEHNINGER, A. L. J. Biol. Chem. **190**, 339 (1951).
7. NOVIKOFF, A. B., HECHT, L., PODBER, E., and RYAN, J. J. Biol. Chem. **194**, 153 (1952).
8. NELSON, N. J. Biol. Chem. **153**, 375 (1944).
9. ALIVISATOS, S. G. A. Ph. D. Thesis, Department of Biochemistry, McGill University, Montreal. 1951.
10. WARBURG, O. and CHRISTIAN, W. Biochem. Z. **303**, 40 (1939).
11. ALBAUM, H. G., CAYLE, T., and SHAPIRO, A. J. Clin. Invest. **30**, 525 (1951).
12. HEVESY, G. and ATEN, A. W. W., Jr. Danske Videnskab. Selskab. Biol. Medd. **14**, 5 (1939). *Cited by* C. B. Mueller and A. B. Hastings. J. Biol. Chem. **189**, 881 (1951).
13. SZEKELY, M., MANYAI, S., and STRAUB, F. B. Acta Physiol. Acad. Sci. Hung. **4**, 31 (1953).
14. RUBINSTEIN, D., KASHKET, S., and DENSTEDT, O. F. Can. J. Biochem. and Physiol. **34**, 61 (1956).
15. GOURLEY, D. R. H. Arch. Biochem. Biophys. **40**, 1 (1952).
16. PRANKERD, T. A. J. and ALTMAN, K. I. Biochem. J. **58**, 622 (1954).
17. BARTLETT, G. R., SAVAGE, E., HUGHES, L., and MARLOW, A. A. J. Appl. Physiol. **6**, 51 (1953).
18. LEUTHARDT, F. and BRUTTIN, H. Helv. Chim. Acta, **35**, 464 (1952); Chem. Abstr. **46**, 6681*c* (1952).
19. SIEKEVITZ, P. and POTTER, V. R. J. Biol. Chem. **215**, 237 (1955).
20. SVENTSITSKAYA, M. B. Bull. Eksptl. Biol. Med. **37**, 32 (1954); Chem. Abstr. **48**, 8284*h* (1954).
21. SURGENOR, D. M., HUNTER, M. J., and BROWN, R. K. *In* Blood cells and plasma proteins. *Edited by* J. L. Tullis, Academic Press, Inc., New York. 1953. p. 315.

A CONTROLLED ASSAY OF CLEARING FACTOR, AND ITS APPLICATION IN STUDIES OF THE ROLE OF HEPARIN IN CLEARING FACTOR PRODUCTION[1]

FRANK C. MONKHOUSE AND RUTH G. MACKNESON

Abstract

A method for measuring clearing factor in vitro has been developed. This method makes use of a standard substrate–receptor system obtained by adding constant amounts of a commercial fat emulsion to stored plasma. Best results are obtained when plasma which has been heated to 56° C for 5 minutes and stored at −20° C is used at 50% concentration. A comparison of the production of clearing factor in different species using plasma from different species was made. With all clearing factors, the human and dog plasmas are superior to the other species studied. The results of intravenous and depot injections of heparin are compared.

The intravenous injections cause maximum prolongation of clotting time almost immediately and maximum amounts of clearing factor within 30 minutes. Depot injections stimulate the production of maximum amounts of clearing factor as quickly as intravenous injections, but the maximum effect on the clotting mechanism is delayed for three to four hours.

The implications of these results are discussed. The application of the method in the study of diseases associated with abnormalities in plasma proteins and fat metabolism is suggested.

Introduction

At various times the clearing effort of heparin (6, 1) was first observed, conflicting reports on the ability of the blood plasma of atherosclerotic individuals to clear lipemia have appeared. Some workers (3, 20), who concluded that there was an impairment of clearing in atherosclerotics, made no distinction between the ability to produce clearing factor and the effectiveness of the plasma as a substrate–receptor system. Pilgeram and Rose (14) found that the plasma of atherosclerotics was only 69% as effective a substrate–receptor system as the plasma of normal persons. In contrast, Angervall and Hood (2) were unable to detect any significant difference between atherosclerotics and normals, either in their ability to produce clearing factor, or to provide a substrate–receptor system. The term "substrate–receptor system" is used to indicate that plasma serves a double role: first to provide a lipoprotein complex as substrate and secondly to provide a protein receptor system for the products of the reaction.

Most methods for the estimation of clearing factor involve changes in optical density of a fat substrate following the addition of postheparin plasma or a fraction thereof. Many workers have used as substrate a lipemic plasma obtained from animals or from human subjects three to five hours after they have been given a fat meal. This provides a substrate of variable density which is unsuitable for a quantitative assay. Furthermore the cells of this lipemic blood are frequently lysed to varying extents, rendering the plasma still more unreliable. To avoid these variations, some workers (4, 9, 17)

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Contribution from the Department of Physiology, University of Toronto, Toronto, Ontario.

have used commercial emulsions of fat, others have used chylomicrons obtained from chyle (5) or cream (8) added directly to plasma. While these modifications ensure constant fat content, they retain another variable in the plasma which is added to supply the substrate–receptor system for the reaction. Even with similar clearing factor and constant amount of fat, clearing will vary when different plasmas are used in the system. This may be due to a variation in the amount of substrate–receptor material in the added plasma, or to the presence of inhibitory material (2, 7). We were interested in making quantitative estimations, and other studies of clearing factor, in different species in an effort to understand the effects of heparin. To do this it was necessary to standardize the assay method so that the only variables were the amount and quality of clearing factor.

Materials and Methods

It has been shown (19) that the change in turbidity with a given preparation of clearing factor is dependent on the initial turbidity. Therefore, since natural lipemic plasmas vary greatly in density, they are not suitable substrates for the quantitative assay of clearing factor.

To overcome this difficulty two sources of fat for preparing a standard substrate were investigated: (*a*) chylomicrons separated from lipemic plasma and (*b*) a commercial fat emulsion. Each was reconstituted to a constant density in control plasma.

(a) Chylomicrons.—After fasting for 12–18 hours, a large dog was given a meal of meat supplemented with 5 ml of corn oil per kg of body weight. Three hours later the blood was collected in 1/10th volume of 3.8% sodium citrate. After centrifugation to separate the cells, the lipemic plasma was transferred to plastic tubes, centrifuged at 10,000 r. p.m. for 1 hour at 4° C. The clear plasma was removed and the chylomicrons were then suspended in control plasma to the desired standard concentration.

(b) Commercial fat emulsion.—'Ediol' (21), a commercial fat emulsion containing 50% coconut oil, was diluted 1:20 in distilled water (at which concentration it is stable at 4° C for several months). For use 0.15 ml of this stock solution was suspended in 10 ml of prepared plasma. Both chylomicrons and 'Ediol' formed excellent substrates for the assay of clearing factor. However, owing to variations in concentration and stability of the chylomicrons, the variation in initial turbidity of the substrate was greater than when 'Ediol' was used. For this reason, 'Ediol' was used throughout these experiments.

Preparation of Standard Controlled Plasma

Oxalated plasma from several dogs was pooled, heated to 56° C for 5 minutes, and immediately cooled. After centrifugation and filtration to remove the fibrin, the plasma was stored in 40-ml aliquots at −20° C. A pool of several liters was prepared at one time, and carefully checked with the preceding pool to ensure the continuity of a uniform substrate–receptor

system. Stored at −20° C, the pool is stable for several months. For use an aliquot was thawed quickly, diluted 1:1 with distilled water, and the fat was added.

Standard Clearing Factor

Postheparin plasma from one dog was always used as a standard. The blood was collected 10 minutes after an intravenous injection of 100 units of heparin per kg of body weight. Dog clearing factor was remarkably stable when stored in small aliquots at −20° C.

Thrombin Clotting Time

This test, described in earlier publications (11), consists of adding 0.1 ml of dilute Thrombin Topical (Parke Davis & Co.) to 0.2 ml of citrated plasma and noting the clotting time. This is a highly sensitive method of detecting the presence of heparin. By varying the concentration of thrombin used, estimations can be made over a wide range of heparin levels.

Assay Method

Turbidity was measured as per cent transmission at 500 mμ in a Junior Coleman spectrophotometer. Distilled water was used to set the zero reading. For each assay, four quantities (0.025, 0.05, 0.1, and 0.2 ml) of clearing factor were added to 1.8 ml of fat–plasma mixture, and water to make the volume 2.0 ml. Transmission was read before incubation and after 1 hour in a

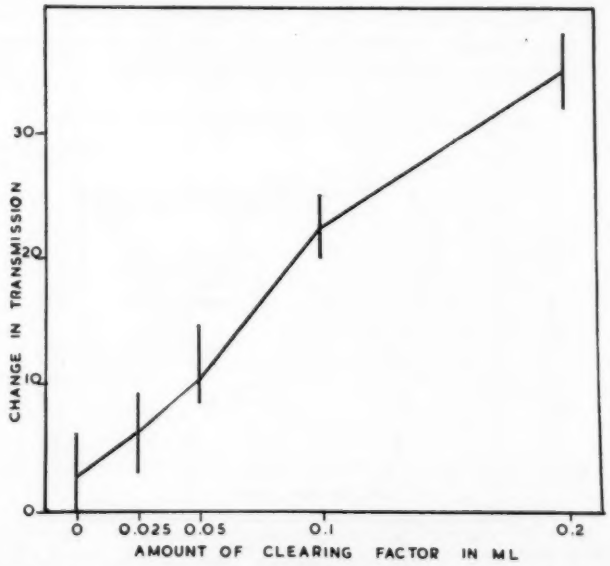

FIG. 1. The change in transmission of standard lipemic plasma following the addition of different quantities of clearing factor. The curve represents the average values for 21 separate assays of a single clearing factor measured over a period of several weeks.

37° C water bath. "Clearing activity" of any quantity was the difference between preincubation and postincubation turbidity readings as measured by per cent transmission.

Figure 1 illustrates the results from 21 assays carried out on separate days on the sample of clearing factor used for control throughout the series of experiments reported in this paper. The values fall on a curve which is sigmoid in nature. We therefore found, when estimating the potency of different clearing factors, that more consistent results were obtained if the values for all four quantities of clearing factor were used. Throughout this paper "clearing factor activity" is expressed as 1/10th of the number obtained when the total clearing activity of the four aliquots is divided by the total volume of these four aliquots. This value represents the *calculated* clearing factor activity of 0.1 ml of the material being assayed and is usually slightly less than the measured value for 0.1 ml. For the 21 consecutive assays, the average clearing factor activity was 19.9 and the standard deviation 1.5.

Results

The Effect of Diluting and Heating Plasma on the Measurement of Clearing Factor

Angervall and Hood (2) report that 40% concentration of human plasma is optimum for the assay of clearing factor. We measured the relationship between plasma concentration and clearing factor activity for dog, rabbit, and human plasmas, and found that 50% is optimal for the comparison of these species and we therefore used this concentration for all subsequent measurements. Considering the differences in the assay system, our results are in good agreement with those of the Swedish workers.

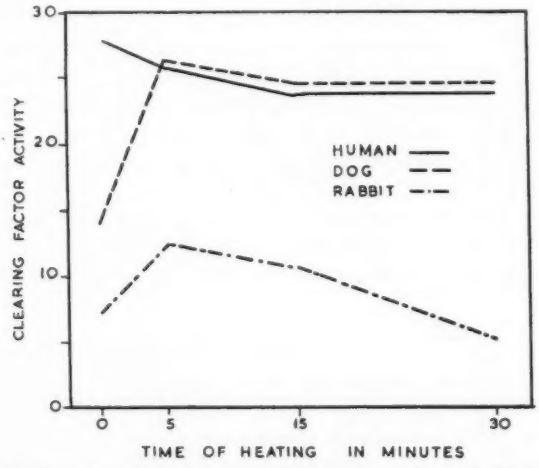

FIG. 2. The effect of heating plasma at 56° C for varying lengths of time on clearing activity. All plasmas are used at 50% concentration. Dog clearing factor is used.

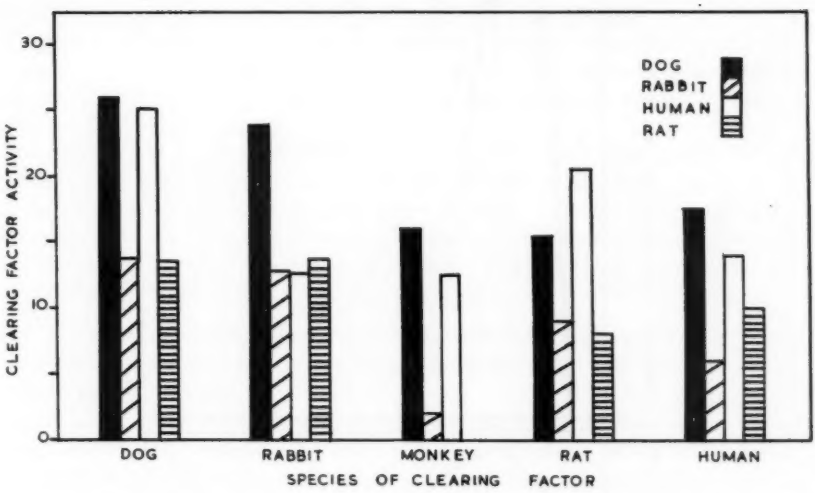

FIG. 3. A comparison of the activity of clearing factor from different species using plasma from different species. All plasmas are used at 50% concentration and heated at 56° C for 5 minutes. The clearing factor used in this experiment was obtained after a dose of heparin calculated to give a maximum response. This varied somewhat in each species and ranged from 100 units/kg in the dog to 300 units/kg in the rabbit.

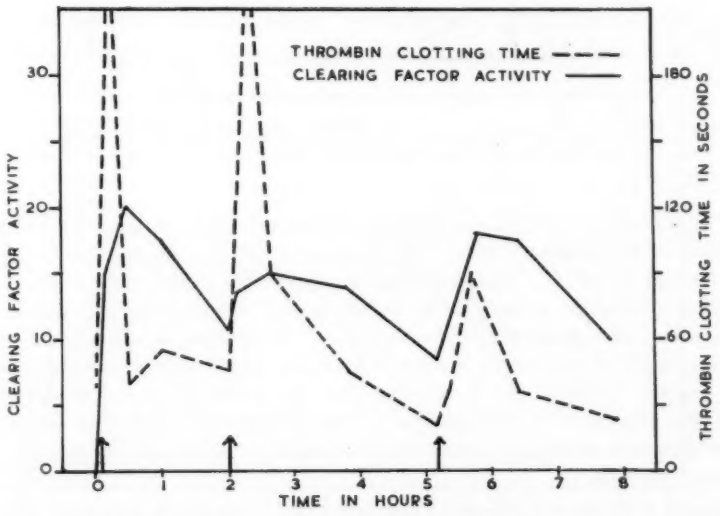

FIG. 4. The effect of repeated intravenous injection of heparin into a dog. Thrombin clotting was carried out by using 1:3 thrombin. Heparin was given in doses of 100 units per kg.

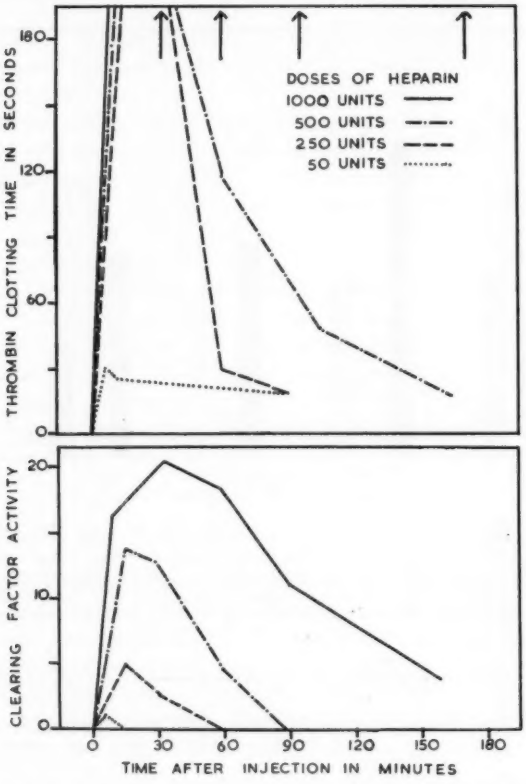

FIG. 5. The effect of varying doses of heparin on the release of clearing factor in the rabbit, and the effect on the thrombin clotting time. Thrombin used at a strength of 1:10.

The decrease in activity in most instances when plasma concentration exceeds 50% is probably due to the presence of inhibitors which are inactivated by heating to 56° C. However, heating also affects efficiency of plasma as a substrate–receptor system. Optimum heating varies with the species, and also with individuals within that species. In studies of the plasma of four normal dogs, three showed marked inhibition, which was removed by heating the plasma to 56° C for 5 minutes, but the other displayed no inhibition, and the efficiency of that plasma decreased directly with time of heating. It is therefore necessary to use pooled plasma in evaluating the species differences of plasma.

Figure 2 shows the effects of heating at 56° C on pools of human, dog, and rabbit plasmas. Heating for 5 minutes appears optimum. Therefore, all plasma used for assay was heated in a water bath at 56° C for 5 minutes, cooled immediately, centrifuged, and stored at 20° C.

Comparison of Clearing Factor and Plasma of Different Species

Figure 3 shows a comparison of different clearing factors on different plasmas. All plasma has been heated to 56° C for 5 minutes, and used at 50% concentration. The activity is recorded as calculated for 0.1 ml of each clearing factor.

In general, the human and dog plasmas appear to be superior sources of a substrate–receptor system, though human plasma is no better than rat or rabbit plasma when rabbit clearing factor is used. Whether the superiority of these plasmas is due to a better fat–protein complex as a substrate or to a superior receptor system is not established. This comparison gives further evidence of the importance of the plasma in estimating clearing factor activity.

The Relationship Between the Route of Heparin Administration and its Effect on Clearing Factor Production and Coagulation of the Blood

Intravenous Injection

(a) In the dog.—Intravenous injection of 100 units of heparin per kg of body weight was sufficient to elicit maximal response of clearing factor. This level of response was maintained for about sixty minutes, but showed a marked decrease during the second hour. This dose causes a rapid and pronounced increase in thrombin clotting time which, however, returns toward normal within 30 minutes. Increase in thrombin clotting time precedes production of clearing factor. With repeated intravenous injections at 2-hour intervals, a similar response to each injection is noted. The results of a typical experiment are shown in Fig. 4.

(b) In the rabbit.—Varying doses of 50 to 1000 units of heparin were injected into rabbits (Fig. 5). In each case the clearing factor appeared later than

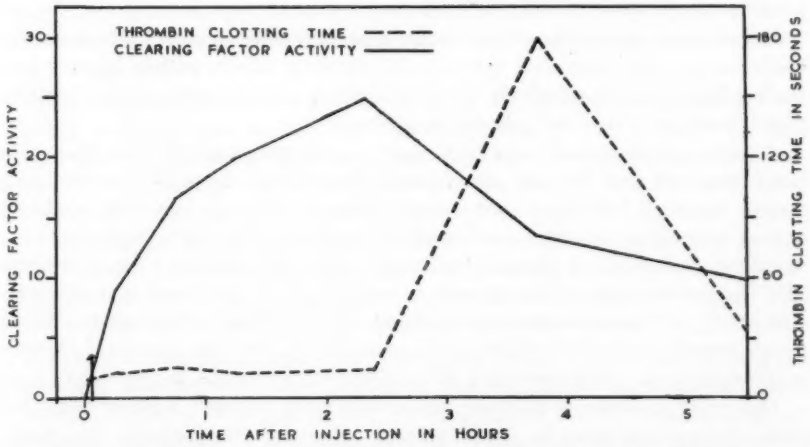

FIG. 6. Clearing factor activity and thrombin clotting time in a dog following a subcutaneous injection of heparin. The dose given was 500 units/kg and the thrombin used for the thrombin clotting time was 1:3. This relatively strong thrombin was used in order to determine maximum heparin levels rather than the presence of heparin.

the effect of heparin on the blood. Repeated intravenous injection of optimal amounts of heparin caused a reaction similar to the initial injection. A much larger dose was required to cause maximal production of clearing factor in the rabbit than in the dog.

Depot Injection (*Subcutaneous or Intramuscular*)

Subcutaneous injection of 500 units per kg of body weight into dogs produced a maximal level of clearing factor which persisted for about six hours. The thrombin clotting time indicates that the heparin did not reach its peak level until three to four hours after the injection, whereas a nearly maximal amount of clearing factor was produced within the first 15 minutes (see Fig. 6). Similar results were obtained in rabbits with intramuscular injection of 1000 units per kg, though the level of clearing factor obtained was much lower in spite of the fact that the blood remained incoagulable for at least 8 hours after the injection of the heparin.

Discussion

From our results it appears that there are at least four factors which may affect the clearing response:

(*a*) The ability to produce clearing factor.

(*b*) The suitability of the plasma for the production of a fat–protein complex as substrate for the clearing reaction (substrate system).

(*c*) The capacity of the plasma to accept the end products of the clearing reaction (receptor system).

(*d*) The amount of inhibitors.

Setting up a standard method for the quantitative assay of clearing factor permits comparisons of the relative roles of these factors.

Intravenous heparin produces maximal prolongation of clotting time almost immediately, and maximal amounts of clearing factor within 30 minutes. Both effects are of relatively short duration, and 2 hours after injection blood levels have returned almost to normal.

Heparin administered subcutaneously or intramuscularly, on the other hand, does not produce any pronounced effect on the thrombin clotting time during the first half hour, but a high level of clearing factor is produced almost as quickly as after intravenous injection. The time lapse between maximal production of clearing factor and thrombin clotting time is presumably the period required for heparin to accumulate in the blood to a measureable level. It has been previously shown (10, 12) that, when heparin levels are measured at intervals following intramuscular injection, a gradual increase in blood heparin which reaches a peak at about four hours, is produced.

The results following intravenous injection of heparin suggest that the clearing factor enters the blood stream only after the heparin has made contact with the peripheral circulation. It is either released from the capillary wall as suggested by Robinson (16), or it is the result of heparin coming into contact with tissues, after passing through the vessel wall.

Following depot injection, however, the heparin comes immediately into contact with the tissues and enters the blood stream gradually by passing into it across the thin-walled capillaries or by way of the lymph. This would permit a considerable amount of clearing factor to enter the blood before the heparin had reached a level sufficient to exert a significant effect on the clotting system.

Regardless of the amount of heparin injected or the method of injection, there is a fairly definite limit to the level of clearing factor obtained. This level is maintained for only a short interval after intravenous injection of heparin but it can be maintained for a considerable period of time when the heparin is deposited as a depot. Nevertheless, even with the latter methods of injection, the clearing factor level often begins to fall while that of heparin is still high. The peak level must obviously be reached when the rate of inactivation of clearing factor is equal to its rate of release or production. So far little experimental work has been done on this aspect of the problem. Spitzer and Spitzer (18) showed that the clearing of oil emulsions by heparinized plasma was decreased after the plasma had been perfused through the excised rat liver. There was also some decrease in activity of clearing factor after incubation with heparinase. Robinson (15) presented evidence to suggest that the stability of clearing factor in vitro was due to the presence of heparin, since it was rapidly inactivated when the heparin was removed by adsorption on a resin column. Our results would indicate that this protective function of heparin is not important in vitro. In the experiments with intravenous injection in the dog, the drop in heparin level is often more rapid than that of the clearing factor, while after intramuscular or subcutaneous injection the level of clearing factor begins to fall even though the concentration of heparin remains high. In the rabbit (Fig. 5) clearing factor disappears from the blood after intravenous injection of heparin while the blood is still incoagulable.

More accurate and specific measurements of the factors which influence the clearing reaction may improve the understanding of the etiology of such pathological conditions as lipoid nephrosis, lipemia of diabetes, and idiopathic hyperlipemia. It has been shown (13) that the lipemia in children suffering from lipoid nephrosis responds very poorly to heparin injection. These patients have a reduced serum albumen level. It may well be that the lipemia which appears in rabbits following repeated bleeding is due to the depletion of the albumen, or to an imbalance in the albumen–globulin ratio. This would interfere with the substrate–receptor system for clearing factor activity.

Recently we had the opportunity of studying the plasma of a depancreatized dog which developed a high degree of lipemia following treatment with orinase. Addition of a potent dog clearing factor to this plasma did not reduce its turbidity. The plasma was centrifuged at 10,000 r.p.m. for 30 minutes, which resulted in the flotation of the lipid and sedimentation of the clear plasma. When the lipid layer was added to standard dog plasma as previously described, the mixture cleared normally. However, when fat

emulsion was added to the clear plasma of that dog, standard clearing factor was inactive. Obviously this animal had a defect in either the ability to form a specific substrate or to form an adequate receptor system. This dog had a low albumen–globulin ratio.

Conclusions

Experiments comparing intravenous and depot injections of heparin lend support to the view that clearing factor is released from the capillary wall or lymphatic vessels. It is also evident that the rate of destruction of clearing factor is rapid. This may explain why it is seldom found in normal plasma and why amounts of heparin, far in excess of what can be considered physiological, are required for its production. Further studies on this aspect of the problem are contemplated.

From a clinical point of view it must be considered that in many instances the conditions which alter the plasma proteins, either absolutely or in relation to each other, may be of primary importance in the action of clearing factor. By centrifugation the low density lipids can be separated from the plasma. Using the techniques outlined in this paper it is then possible to examine the plasma and fat separately. By such screening tests it should be possible to determine those situations in which heparin could be expected to be beneficial.

Acknowledgments

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References

1. Anderson, N. G. and Fawcett, B. Proc. Soc. Exptl. Biol. Med. **74**, 768 (1950).
2. Angervall, G. and Hood, B. Acta Med. Scand. **57**, 407 (1957).
3. Block, W. J., Mann, F. D., and Baker, N. W. Proc. Staff Meetings Mayo Clinic, **26**, 246 (1951).
4. Brown, R. K., Boyle, E., and Anfinsen, C. B. J. Biol. Chem. **204**, 423 (1953).
5. French, J. E., Robinson, D. S., and Florey, H. W. Quart. J. Exptl. Physiol. **38**, 101 (1953).
6. Hahn, P. F. Science, **98**, 19 (1943).
7. Hollet, C. and Meng, H. C. Federation Proc. **16**, 60 (1957).
8. Hood, B. and Angervall, G. Acta Med. Scand. **158**, 13 (1957).
9. Meng, H. C., Hollett, C., and Cole, W. E. Am. J. Physiol. **179**, 314 (1954).
10. Monkhouse, F. C. Am. J. Physiol. **178**, 223 (1954).
11. Monkhouse, F. C. Can. J. Biochem. and Physiol. **33**, 112 (1955).
12. Monkhouse, F. C., MacMillan, R. L., and Brown, K. W. G. J. Lab. Clin. Med. **43**, 92 (1953).
13. Nikkila, E. and Grasbeck, R. Acta Med. Scand. **150**, 39 (1954).
14. Pilgeram, L. O. and Rose, L. C. Circulation, **14**, 502 (1956).
15. Robinson, D. S. Quart. J. Exptl. Physiol. **41**, 195 (1956).
16. Robinson, D. S. and French, J. E. Quart. J. Exptl. Physiol. **42**, 151 (1957).
17. Shore, B., Nichols, A. V., and Freeman, N. K. Proc. Soc. Exptl. Biol. Med. **83**, 216 (1953).
18. Spitzer, J. A. and Spitzer, J. J. Am. J. Physiol. **185**, 18 (1956).
19. Williams. G. R. Biochim. et Biophys. Acta, **13**, 72 (1954).
20. Zemplenyi, T., Felt, V., Fodor, J., Reichl, D., and Pilar, J. Chem Abstr. **50**, 2051 (1956).
21. Zuckermann, L. and Singher, H. O. Proc. Soc. Exptl. Biol. Med. **89**, 315 (1955).

MECHANISM OF ACTION OF THE LACTIC DEHYDROGENASE OF THE MAMMALIAN ERYTHROCYTE

I. INFLUENCE OF INHIBITORS[1]

PAUL OTTOLENGHI AND ORVILLE F. DENSTEDT

Abstract

This paper pertains to the inhibition of lactic dehydrogenase by substances structurally related to its substrates, pyruvate and lactate. Oxalate, tartronate, and malonate inhibit the enzyme in a competitive manner towards lactate, and in a noncompetitive manner towards pyruvate. Phenoxyacetate causes simultaneous competitive and noncompetitive inhibition towards both lactate and pyruvate. The results obtained lead to the conclusion that, for their activation, pyruvate and lactate react with different sites on the enzyme surface.

The inhibition of lactic dehydrogenase by various substances structurally related to its substrates, lactate and pyruvate, has been studied by several workers. Quastel and Wooldridge (1) in 1928 tested about 30 compounds as probable inhibitors of lactic acid oxidation with *E. coli* as the source of the enzyme. The compounds found to cause the strongest inhibition were glycolic, oxalic, tartronic, mandelic, mesotartaric, and parabanic acids. In the same year Bernheim (2) demonstrated the inhibition of the lactic dehydrogenase of yeast by oxalic, glyceric, and pyruvic acids. The more recent studies by Neilands (3) with the lactic dehydrogenase of heart muscle have confirmed the earlier findings with oxalate and have shown that acetaldehyde, acetone, and methyllactate do not inhibit the enzyme. Neilands found, contrary to the earlier findings of Quastel *et al.* (1), that glycolic acid causes no inhibition of the enzyme from heart muscle (3). Hakala *et al.* (4) have reported that oxamic acid is an inhibitor of the enzyme, and Winer *et al.* (5) have found the inhibition to be competitive with respect to pyruvate, but noncompetitive with respect to DPNH.[2]

The present communication describes a more detailed study of the mechanism of inhibition of lactic dehydrogenase of the erythrocyte by some substances structurally related to lactic and pyruvic acids. The phenomenon of substrate inhibition, i.e. the inhibition of the enzymatic reduction of pyruvate by a high concentration of pyruvate, will be discussed in another paper (6).

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Contribution from the Department of Biochemistry, McGill University, Montreal, Quebec. This study was supported by the Defence Research Board of Canada, Grant No. 9350-01, Project D 50-93050-01. The work presented here was presented by one of the authors (P.O.) to the Faculty of Graduate Studies and Research, McGill University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2]The following abbreviations have been used: DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; tris, trishydroxymethylaminomethane; E, free enzyme; S, substrate; C, coenzyme; I, inhibitor; P, products; ES, EI, etc., complexes of enzyme with substrate, with inhibitor, etc.; K_i, K'_i, dissociation constants of enzyme–inhibitor complexes; K_s, K_c, dissociation constants of enzyme–substrate and enzyme–coenzyme complexes; v, velocity of the reaction; v_m, maximum velocity of the reaction.

Experimental

Reagents

All the reagents were commercial preparations with the exception of the reduced DPN, which was prepared from DPN by reduction with ethanol and alcohol dehydrogenase (7).

Enzyme Preparation

The lactic dehydrogenase for the studies was prepared from rabbit erythrocytes. That these cells provide a rich source of the enzyme was shown by Quastel and Wheatley in 1938 (10), and later by Alivisatos and Denstedt (9), who segregated the enzyme in the soluble fraction of the cell.

Blood was drawn from a rabbit into a heparin solution in isotonic KCl and the erythrocytes were washed several times with isotonic KCl. They were then hemolyzed by freezing and thawing of the sample, the treatments being repeated three times. The stroma residue was removed by centrifugation. The supernatant stroma-free hemolyzate, diluted with isotonic KCl to a concentration which would give a suitable reaction rate, was used as the enzyme preparation.

Method of Assay

The enzymatic activity was measured in terms of the rate of change in the concentration of the reduced coenzyme (DPNH) in the reaction medium. The concentration of DPNH, in turn, was estimated from the optical density of the medium at a wavelength of 340 mμ (8), with a Beckman DU spectrophotometer equipped with a photomultiplier.

The reaction mixture included a phosphate or a tris buffer, the enzyme preparation, substrate (pyruvate or D-lactate[3]), coenzyme (DPN or DPNH) and, when desired, an inhibitor. All the ingredients except the coenzyme were placed in the cuvette and the final total volume of the reaction mixture (including the coenzyme) was 2.5 ml. The reaction was carried out at a temperature of 25° C.

The reaction was started by adding desired concentrations of the coenzyme. The contents of the cuvette were mixed by inversion and the first reading was taken immediately. Further readings were taken at regular intervals during the period of the zero-order phase of the reaction.

Results

Several substances with a hydroxyl, ketone, or carboxyl group, or a combination of these functional groups, were tested for possible inhibitory influence on the activity of the lactic dehydrogenase preparation. The results are indicated in Table I.

The inhibition obtained with tartronate, malonate, and oxalate was of the same type. These compounds act in a competitive manner when tested

[3]D-Lactate is as good a substrate as L-lactate, for the lactic dehydrogenase of the rabbit erythrocyte, and offers the advantage that it is inferior to the latter as a nutrient for bacterial growth during storage of the reagent.

against lactate but in a noncompetitive manner with pyruvate as the substrate. The dissociation constants (K_i) for the enzyme–inhibitor complexes formed are indicated in Table II. Oxalate was chosen as a representative of the three inhibitors mentioned above and its effect on the enzyme with DPN as the variable substrate and with a constant concentration of lactate was studied.

Caution must be exercised in the determination of the kinetic constants of enzymes such as lactic dehydrogenase, which requires the formation of a complex between the enzyme and the coenzyme (DPN) before the substrate (lactate) can combine with the enzyme (10). In plots of the reciprocal of the reaction velocity against the reciprocal of the concentration of DPN, the reciprocal of the maximum velocity is not equivalent to the intercept on the y-axis nor is the Michaelis constant for DPN equivalent to the negative reciprocal of the intercept on the x-axis.

TABLE I

INFLUENCE OF VARIOUS COMPOUNDS ON THE ACTIVITY OF LACTIC DEHYDROGENASE

Compound tested	Molar concentration	Substrate	Inhibition
Acetone	1.6×10^{-2}	Pyruvate	−
Isopropanol	2×10^{-2}	Pyruvate	−
Acetate	1.6×10^{-2}	Pyruvate	−
Propionate	1.2×10^{-1}	Pyruvate	−
Oxalate	—	Pyruvate	+
		Lactate	+
		DPN	+
Malonate	—	Pyruvate	+
		Lactate	+
Succinate	3×10^{-2}	Pyruvate	−
Tartronate	—	Pyruvate	+
		Lactate	+
Phenylacetate	6×10^{-2}	Pyruvate	−
Mandelate	6×10^{-2}	Pyruvate	−
Phenoxyacetate	—	Pyruvate	+
		Lactate	+

NOTE: + denotes occurrence of inhibition, − absence of inhibition.

TABLE II

K_i VALUES FOR INHIBITORS OF LACTIC DEHYDROGENASE

Inhibitor	Concentration and pH of substrate			
	Pyruvate	pH	Lactate	pH
Tartronate	4.8×10^{-4} M	8.0*	2.6×10^{-3} M	8.0†
Malonate	2.7×10^{-2} M	8.0†	6.4×10^{-3} M	8.0†
Oxalate	7.0×10^{-5} M	7.0*	2.2×10^{-4} M	8.5*

* 8×10^{-2} M phosphate buffer.
† 4×10^{-2} M tris buffer.

The mathematical expression which describes the reaction mechanism is:

$$[1] \qquad \frac{1}{v} = \frac{1}{v_m} + \frac{K_s}{v_m\,[S]} + \frac{K_s\,K_c}{v_m\,[S]\,[C]}.$$

When the values of $1/v$ are plotted against those of $1/[C]$ for several concentrations of S, the points will lie on straight lines which intersect at a common point in the second quadrant, with coordinates $(-1/K_c, v_m)$.

In the presence of an inhibitor, I, which competes with S for a site on the enzyme (e.g. competition between oxalate and lactate), equation [1] becomes:

$$[2] \qquad \frac{1}{v_i} = \frac{1}{v_m}\left(1 + \frac{K_s}{[S]} + \frac{K_s\,[I]}{[S]\,K_i}\right) + \frac{K_sK_c}{v_m\,[S]\,[C]}.$$

A plot of $1/v_i$ against $1/[C]$ for various concentrations of I will yield a series of parallel lines with a slope of $\frac{K_sK_c}{v_m\,[S]}$ and intercepts on the y-axis of values corresponding to

$$\frac{1}{v_m}\left(1 + \frac{K_s}{[S]} + \frac{K_s\,[I]}{[S]\,K_i}\right).$$

These relationships correspond to the uncompetitive type of inhibition.

As the value of the concentration of S approaches infinity, the velocity of the reaction in the presence of inhibitor will approach the maximum. The intercept on the y-axis then assumes the value $1/v_m$ which is identical with that obtained in the absence of the inhibitor.

From Fig. 1 it is apparent that the maximum velocity of the reaction remains unchanged even after the addition of the inhibitor to the system. From Fig. 2 (which indicates the results obtained with two concentrations of the inhibitor) and with the application of equation [2], the K_i for oxalate at pH 9.2 was found to be $2.1\times10^{-4}\,M$. In Fig. 2, the line which corresponds to an infinite concentration of lactate was obtained from the values of v_m and K_c as found by extrapolation of the lines in Fig. 1.

Although the exact mechanisms that underlie the phenomenon of specificity in enzyme catalysis are obscure, the concept of specific sites on the enzyme surface for the attachment of the substrates is a reasonable one since it satisfies the experimental findings. Whatever the mechanism may be by which the complex formation occurs it is clear that the properties of the functional groups and the peculiar over-all properties of the substrates, coenzymes, and inhibitors are fundamentally involved in determining the affinity and specificity of the active catalytic system for the above-mentioned substances. In the present discussion we use the commonly held concept of the presence of specific sites on the enzyme surface where activation of the substrates or reaction with inhibitors occurs.

The evidence referred to in the preceding paragraphs appears to indicate the existence on the dehydrogenase of two specific sites, one for the attachment of lactate and the other for pyruvate, since the three inhibitor substances tartronate, malonate, and oxalate compete specifically with lactate for its site on the enzyme but not with pyruvate for its site. When phenoxyacetate

was tested as an inhibitor and the double-reciprocal (Lineweaver–Burk) plots (11) of the data were made, a most unexpected result was obtained, namely, that this compound appeared to act as a competitive inhibitor of both lactate and pyruvate. As this implication was inconsistent with our hypothesis, we were obliged to reanalyze the results in accordance with the alternative hypothesis, that the inhibitor is capable of forming a complex with both of the substrate-binding sites on the enzyme surface. On the

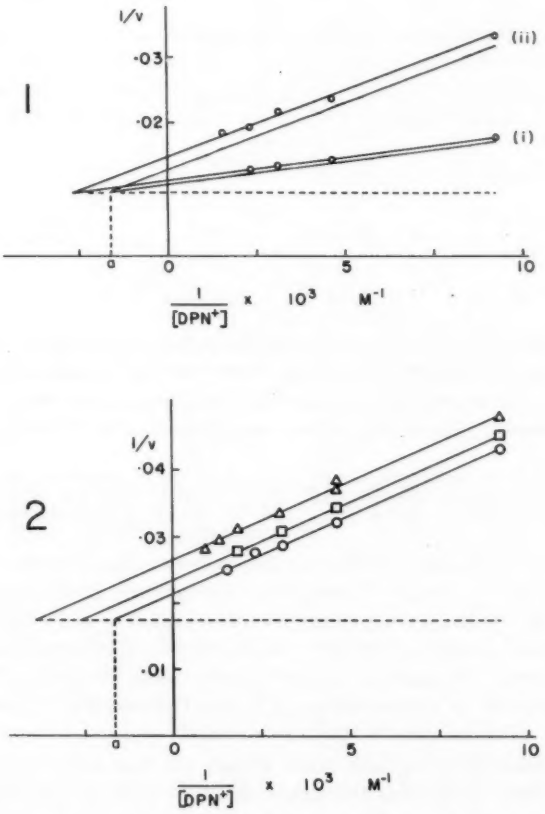

FIG. 1. The inhibition of the lactate–DPN system by oxalate. Substrate: DPN. Buffer: 4×10^{-2} *M* tris, pH 9.2. D-Lactate concentration: (i) $6\times10^{-3}M$; (ii) $2\times10^{-3}M$. Oxalate concentration:—, 0; ○, 5×10^{-5} *M*. Value of a, $-1/K_e$ (for DPN). Horizontal broken line represents the theoretical plot obtained with infinite concentration of DPN. Inhibition is of the uncompetitive type.

FIG. 2. The inhibition of the lactate–DPN system by oxalate. Substrate: DPN. Buffer: 4×10^{-2} *M* tris, pH 9.2. D-Lactate concentration: 4×10^{-3} *M*. Oxalate concentration: ○, 0; □, 10^{-4} *M*; △, 3×10^{-4} *M*. Value of a, $-1/K_e$ (for DPN). Horizontal broken line represents the theoretical plot obtained with infinite concentration of DPN. Inhibition is of the uncompetitive type.

basis of this postulate both the competitive and the noncompetitive modes of inhibition would occur simultaneously. The forms in which the enzyme could exist, and the possible reactions between the enzyme, the substrate, and the inhibitor, are represented by the following equations:

[3] $$E + I \overset{K_i}{\rightleftharpoons} EI$$

[4] $$E + I \overset{K'_i}{\rightleftharpoons} EI'$$

[5] $$ES + I \overset{K'_i}{\rightleftharpoons} ESI'$$

[6] $$EI' + S \overset{K_s}{\rightleftharpoons} ESI'$$

[7] $$EI + I \overset{K'_i}{\rightleftharpoons} EII'$$

[8] $$EI' + I \overset{K_i}{\rightleftharpoons} EII'$$

[9] $$E + S \overset{K_s}{\rightleftharpoons} ES \rightarrow E + P$$

where ES represents the activated enzyme–substrate complex; EI the competitive enzyme–inhibitor association, with K_i the dissociation constant; and EI′ the noncompetitive enzyme–inhibitor association with K'_i the dissociation constant. From the above relationships the following expression is obtained:

[10] $$\frac{1}{v_i} = \frac{1}{v_m}\left(1 + \frac{[I]}{K'_i}\right) + \frac{K_s}{v_m}\left(1 + \frac{[I]}{K_i} + \frac{[I]}{K'_i} + \frac{[I]^2}{K_iK'_i}\right)\frac{1}{[S]}.$$

The plots of $1/v_i$ against $1/[S]$ for various concentrations of the inhibitor in such a system give a series of lines with no common point of intersection. It is possible for the lines to converge towards a region in the second quadrant, close to the y-axis, in such a way that the occurrence of competitive inhibition would be implied. By using a variant of the usual Michaelis–Menten (12) equation as applied by Augustinsson (13) and Hofstee (14, 15), an interpretation can be obtained which fully accounts for the experimental findings.

The above-mentioned authors have shown (as had been done before by Haldane and Stern (16)) that the simple Michaelis–Menten equation can be stated as follows:

[11] $$v = v_m - K_s v/[S].$$

On plotting the values of v against the corresponding values of $v/[S]$ we obtained a line with a slope of $-K_s$ and an intercept on the y-axis, of v_m. Analogous equations which correspond to the competitive and noncompetitive types of inhibition also can be derived. In the case of the competitive type of inhibition a plot of v_i against $v_i/[S]$ will give a series of straight lines with

a common intercept on the y-axis and with increasing negative slopes for increasing concentrations of the inhibitor. With the noncompetitive mode of inhibition the slope of the lines remains constant with increase in the concentration of the inhibitor but the intercept on the y-axis decreases. However, when both the competitive and the noncompetitive types of inhibition occur simultaneously the situation can be represented by the following rearrangement of equation [10]:

$$[12] \qquad v_i = \frac{v_m \; K'_i}{K'_i + [I]} - \frac{v_i}{[S]} \left(1 + \frac{[I]}{K_i}\right) K_s.$$

From equation [12] it is obvious that both the slope and the intercept on the y-axis are dependent upon the concentration of the inhibitor.

By plotting the values of v_i against those of $v_i/[S]$ as indicated in Figs. 3 and 4, it is apparent that phenoxyacetate does indeed behave in this duplex

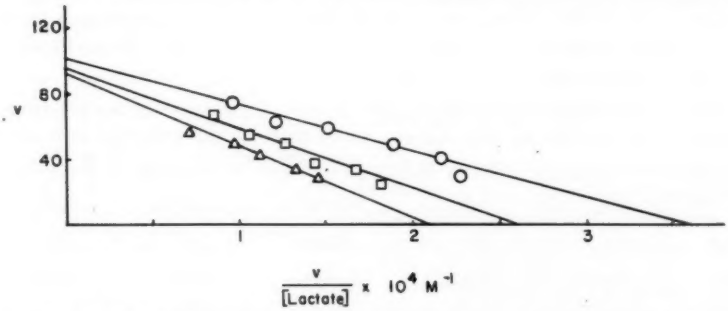

FIG. 3. The inhibition of the lactate–DPN system by phenoxyacetate. Substrate: D-lactate. Buffer: 4×10^{-2} M tris, pH 8.0. DPN concentration: 2.2×10^{-3} M. Phenoxyacetate concentration: ○, 0; □, 10^{-2} M; △, 2×10^{-2} M. Inhibition is of both the competitive and the noncompetitive types.

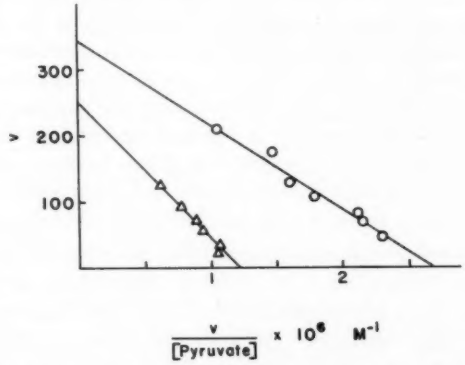

FIG. 4. The inhibition of the pyruvate–DPNH system by phenoxyacetate. Substrate: pyruvate. Buffer: 4×10^{-2} M tris, pH 8.0. DPNH concentration: 10^{-4} M. Phenoxyacetate concentration: ○, 0; △, 2×10^{-2} M. Inhibition is of both the competitive and the noncompetitive types.

manner. One may conclude, therefore, that phenoxyacetate can combine with both the site at which lactate is normally bound and that at which pyruvate is bound. The values of the K_i and the K'_i were estimated from the slope and the intercept on the y-axis of the plot corresponding to the case without the inhibitor and from the analogous parameters of the plots obtained in the presence of the inhibitor. The values obtained for these constants are given in the footnotes which accompany the figures.

Discussion

The study of the influence of inhibitors on the interconversion of lactate and pyruvate, as catalyzed by lactic dehydrogenase, has yielded information concerning the mode of activation of the substrates by the enzyme. The nature of the functional groups on the substrates which might be involved in the formation of the complex with the enzyme molecule to bring about the activation of the substrates, and the exchange of two hydrogen ions and two electrons between the substrate and coenzyme, may be deduced from the results presented.

From a comparison of the structures of the inhibitors which were found to compete with lactate for attachment to its site on the enzyme, it is evident that the only group which is common to all the compounds is the carboxyl.

CH_3	CH_3	OH	COOH	COOH	O–Ph
CHOH	CO	CO	CHOH	CH_2	CH_2
COOH	COOH	COOH	COOH	COOH	COOH
Lactic acid[4]	Pyruvic acid[5]	Oxalic acid	Tartronic acid	Malonic acid	Phenoxy-acetic acid

It is very probable, therefore, that the carboxyl group of the lactate becomes bound to the enzyme during the formation of the enzyme–lactate complex. The carboxyl group alone, however, is not sufficient as was indicated by the failure of acetic and propionic acids to combine with the enzyme. It would appear, therefore, that another functional group in an adjacent position is essential for the formation of a complex with the enzyme, to occur. Oxalic acid, with vicinal carboxyl groups, is a potent inhibitor of the lactic dehydrogenase. The introduction of a methylene group between the two carboxyls (as in malonic acid) reduces the inhibitory influence while the introduction of a second methylene group (as in succinic acid) abolishes it altogether.

Since the carboxyl group is most probably involved in the formation of a complex between lactate and the enzyme, the ketone group of pyruvate remains the most likely group involved in the formation of a complex between the enzyme and this substrate. As with the carboxyl group, however, the

[4]Lactic acid is included for comparison.

[5]Inhibition of the pyruvate-to-lactate reaction by a high concentration of pyruvate was shown (6) to occur by the formation of an enzyme–pyruvate complex in which the inhibitory molecule of pyruvate is attached to the lactate-binding site.

presence of a carbonyl alone is not sufficient to permit the binding of a molecule to the lactic dehydrogenase. Oxalic acid may be expected to possess some properties of an α-keto acid, but this acid manifests no competition with pyruvic acid for the same specific site on the enzyme. The fact that the carbonyl group of oxalic acid is part of a carboxyl group may indeed prevent the binding of oxalic acid at the pyruvate-binding site on lactic dehydrogenase.

Phenoxyacetate has been shown to compete with both lactate and pyruvate. Although phenoxyacetate contains an ether linkage, it shows, according to Fisher-Hirschfelder-Taylor atom models, an atomic arrangement similar to that in pyruvate. In both these compounds the oxygen attached to the carbon in the α-position with respect to the carboxyl has two pairs of unshared electrons as well as a residual area of electronegativity imposed by the resonance effects of the adjacent groups. These structural similarities would appear to account for the competition between pyruvate and phenoxyacetate for the pyruvate-binding site.

References

1. QUASTEL, J. H. and WOOLDRIDGE, W. R. Biochem. J. **22**, 689 (1928).
2. BERNHEIM, F. Biochem. J. **22**, 1178 (1928).
3. NEILANDS, J. B. J. Biol. Chem. **208**, 225 (1954).
4. HAKALA, M. T., GLAID, A. J., and SCHWERT, G. W. J. Biol. Chem. **221**, 191 (1956).
5. WINER, A. D., NOVOA, W. B., and SCHWERT, G. W. J. Am. Chem. Soc. **79**, 6571 (1957).
6. OTTOLENGHI, P. and DENSTEDT, O. F. Can. J. Biochem. and Physiol. (Paper No. 2 of the present series). This issue.
7. Pabst Laboratories Circular OR–10. Ultraviolet absorption of 5′-nucleotides. 1956. p. 18.
8. VON EULER, H., ADLER, E., GUNTHER, G., and HELLSTROM, H. Z. physiol. Chem. Hoppe-Seyler's, **245**, 217 (1937).
9. ALIVISATOS, S. G. A. and DENSTEDT, O. F. Science, **114**, 281 (1951).
10. QUASTEL, J. H. and WHEATLEY, A. H. M. Biochem. J. **32**, 936 (1938).
11. LINEWEAVER, H. and BURK, D. J. Am. Chem. Soc. **56**, 658 (1934).
12. MICHAELIS, L. and MENTEN, M. L. Biochem. Z. **49**, 333 (1913).
13. AUGUSTINSSON, K. B. Acta Physiol. Scand., Suppl. **52**, 15 (1948).
14. HOFSTEE, B. H. J. Science, **116**, 329 (1952).
15. HOFSTEE, B. H. J. Enzymologia, **17**, 273 (1956).
16. HALDANE, J. B. S. and STERN, K. G. Allgemeine Chemie der Enzyme, Leipzig. 1932. p. 119. *Quoted by* J. B. S. Haldane in Nature, **179**, 832 (1957).

MECHANISM OF ACTION OF THE LACTIC DEHYDROGENASE OF THE MAMMALIAN ERYTHROCYTE

II. THE MECHANISM OF SUBSTRATE INHIBITION[1]

PAUL OTTOLENGHI AND ORVILLE F. DENSTEDT

Abstract

The inhibition of lactic dehydrogenase produced by an excess of the substrate pyruvate has been shown to be of the noncompetitive type. Thus, two sites on the enzyme appear to be involved: one for the activation of pyruvate, and a second one which, when complexed with a molecule of pyruvate, renders the enzyme inactive.

The enzymatic reduction of pyruvate to lactate as represented in equation [1] is inhibited in the presence of a high concentration of pyruvate (1, 2, 3).[2]

$$[1] \qquad \text{Pyruvate} + \text{DPNH} + \text{H}^+ \rightleftharpoons \text{lactate} + \text{DPN}^+.$$

This inhibition affords an example of the inhibition of an enzymatic reaction by an excess of the substrate.

Numerous examples of this type of inhibition have been described and various hypotheses have been suggested by Haldane (4), Lineweaver and Burk (5), and others to explain the mechanism of the substrate interference. All the hypotheses are based on the assumption that the enzyme tends to combine with more than one molecule of substrate to form an inactive complex. Kalow *et al.* (6) have shown that the inhibition of serum cholinesterase by the substrate benzoylcholine cannot be explained on the basis of the above-mentioned hypotheses. It can be accounted for, however, by another mechanism also proposed by Lineweaver and Burk (5), namely that an enzyme–multisubstrate complex is formed in the presence of an excess of the substrate, and that the rate of its breakdown is slower than that of the complex of the enzyme with only one molecule of the substrate. The present authors, however, believe that it is not necessary to make this assumption to explain the inhibition of lactic dehydrogenase by pyruvate.

Theoretical Considerations

Hofstee (7) has suggested that the inhibition by substrate might occur in either of the following ways: (*a*) in a competitive manner, by attachment of

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[2]The following abbreviations have been used: DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; E, free enzyme; S, substrate; P, products; I, inhibitor; ES_1, ES_1S_2, ES_1I, etc., complexes of enzyme with substrate or inhibitor, etc.; K_s, K_{ss}, K_i, dissociation constants; v, v_i, velocity of the reaction in the absence and in the presence of inhibitor; v_m, maximum velocity of the reaction.

a second molecule of the substrate at the same site at which the first (activated) molecule is attached thus forming an inactive complex, or, (*b*) in a noncompetitive manner, by attachment of a second substrate molecule at a different site from that which normally activates the substrate. The following reactions therefore are possible:

[2] $$E + S \overset{K_s}{\rightleftharpoons} ES_1 \rightarrow P + E,$$

[3] $$E + S \overset{K_{ss}}{\rightleftharpoons} ES_2,$$

[4] $$ES_1 + S \overset{K_{ss}}{\rightleftharpoons} ES_1S_2,$$

[5] $$ES_2 + S \overset{K_s}{\rightleftharpoons} ES_1S_2,$$

where ES_1 and ES_2 are different enzyme–substrate interactions and ES_1 is the only complex of the enzyme and substrate that can give rise to products. If inhibition by the substrate is noncompetitive, all the reactions from [2] to [5] inclusive are possible, but if it is competitive, reactions [3] and [5] cannot occur.

With these assumptions the above described equations can be solved in terms of the active complex, ES_1, to give the following equations:

[6] $$\frac{v_m}{v} = 1 + \frac{K_s}{[S]} + \frac{K_s}{K_{ss}} + \frac{[S]}{K_{ss}}$$

for the noncompetitive case, and

[7] $$\frac{v_m}{v} = 1 + \frac{K_s}{[S]} + \frac{[S]}{K_{ss}}$$

for the competitive case.

To ascertain the mechanism by which a high concentration of the substrate produces an inhibition of the enzymatic reaction, an experimental method suggested by Hofstee can be applied (7). This method involves the use of an inhibitor of the enzyme along with a high concentration of the inhibiting substrate. Either a competitive or a noncompetitive inhibitor can be used. The experiments to be reported in this communication were carried out with oxalate. This compound causes strong inhibition of lactic dehydrogenase (2, 8, 9) and we have shown that the inhibition towards the substrate pyruvate is of the noncompetitive type (9). Only the relations with reference to this type of inhibitor will be considered.

The following are the possible reactions in a system which contains a noncompetitive inhibitor along with a substrate capable of acting as an inhibitor:

[8] $$E + S \overset{K_s}{\rightleftarrows} ES_1 \rightarrow E + P,$$

[9] $$E + S \overset{K_{ss}}{\rightleftarrows} ES_2,$$

[10] $$ES_1 + S \overset{K_{ss}}{\rightleftarrows} ES_1S_2,$$

[11] $$ES_2 + S \overset{K_s}{\rightleftarrows} ES_1S_2,$$

[12] $$E + I \overset{K_i}{\rightleftarrows} EI,$$

[13] $$ES_1 + I \overset{K_s}{\rightleftarrows} ES_1I,$$

[14] $$ES_2 + I \overset{K_i}{\rightleftarrows} ES_2I,$$

[15] $$ES_1S_2 + I \overset{K_i}{\rightleftarrows} ES_1S_2I,$$

[16] $$EI + S \overset{K_s}{\rightleftarrows} ES_1I,$$

[17] $$EI + S \overset{K_{ss}}{\rightleftarrows} ES_2I,$$

[18] $$ES_1I + S \overset{K_{ss}}{\rightleftarrows} ES_1S_2I,$$

[19] $$ES_2I + S \overset{K_s}{\rightleftarrows} ES_1S_2I,$$

where the complex ES_1 is the only one capable of giving rise to products. One of the following three distinct cases is possible in the system as specified:

Case *a*, in which the two substrate molecules combine with the enzyme at the same site, while the inhibitor attaches to an auxiliary site. In this situation the reactions [9], [11], [14], [17], and [19] are not relevant.

Case *b*, in which the inhibiting molecule of substrate competes with the inhibitor for the same site on the enzyme. Under this condition reactions [14], [15], [17], [18], and [19] are not relevant.

Case *c*, in which the inhibitor and the two substrate molecules are attached at distinct sites on the enzyme. In this case all the reactions described above will occur.

Case *a* corresponds to the condition of competitive substrate inhibition, while cases *b* and *c* represent noncompetitive situations.

In case *a*, by expressing the concentration of each of the possible complexes in terms of the active complex, ES_1, the following expression is obtained:

[20] $$\frac{v_m}{v_i} = \left(1 + \frac{K_s}{[S]} + \frac{[S]}{K_{ss}}\right)\left(1 + \frac{[I]}{K_i}\right).$$

By expressing equations [7] and [20] in terms of v_m, and equating the two expressions, one obtains the relation:

[21] $$v - v_i = v - \left(\frac{v - v_i}{[I]}\right) K_i.$$

A plot of $(v-v_i)$ against $(v-v_i)/[I]$ will give a straight line with an intercept of v on the y-axis and a slope of $-K_i$, i.e. the slope is independent of the concentration of the substrate.

For case *b*,

[22] $$\frac{v_m}{v_i} = \left(1 + \frac{K_s}{[S]}\right)\left(1 + \frac{[I]}{K_i}\right) + \frac{K_s}{K_{ss}} + \frac{[S]}{K_{ss}}.$$

Proceeding as in the previous case, and equating expressions [6] and [22], we obtained the following:

[23] $$(v - v_i) = v - \left(\frac{v - v_i}{[I]}\right)\left(1 + \frac{[S]}{K_s}\right) K_i.$$

In this case a plot of $(v-v_i)$ against $(v-v_i)/[I]$ will give a line with a slope of

$$- K_i \left(1 + \frac{[S]}{K_{ss}}\right).$$

The value of the slope is now obviously dependent upon the concentration of the substrate. If another plot be made of the negative slope of equation [23] as obtained with various concentrations of the substrate S, against the corresponding concentrations of S, the line obtained will have a constant slope of value K_i/K_{ss}, and an intercept on the y-axis equal to K_i.

For case *c*,

[24] $$\frac{v_m}{v_i} = \left(1 + \frac{K_s}{[S]} + \frac{[S]}{K_{ss}} + \frac{K_s}{K_{ss}}\right)\left(1 + \frac{[I]}{K_i}\right).$$

By equating expressions [6] and [24] one obtains an equation identical with [21].

Thus, with the use of a noncompetitive inhibitor such as oxalate it is not possible to differentiate mechanism *a* from mechanism *c*, but *b* can be distinguished from the other two.

Experimental

Materials and Methods

The velocity of the reaction was measured in terms of the rate of change of the optical density at a wavelength of 340 mμ as previously described (9). The reaction mixture included 8×10^{-2} *M* phosphate buffer of pH 7.0, 10^{-4}

M DPNH, the enzyme, sodium pyruvate, and sodium oxalate, in the concentrations specified in the text. The final volume of the reaction mixture was 2.5 ml and the reaction was carried out at 25° C.

Results

The curves in Fig. 1 indicate the effect of the variation of the substrate concentration on the activity of the enzyme. The value of K_s for pyruvate, as obtained from the double reciprocal plot for the range of pyruvate concentration at which there was no inhibition, was found to be 4×10^{-5} M.

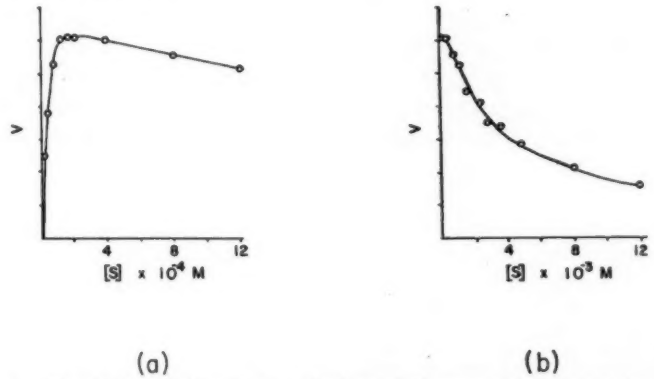
To determine the value of the K_{ss}, equations [6] and [7] may be rearranged as follows;

[25] $$\frac{v_m}{v} - \frac{K_s}{[S]} = 1 + \frac{K_s}{K_{ss}} + \frac{[S]}{K_{ss}}$$

for the noncompetitive case, and

[26] $$\frac{v_m}{v} - \frac{K_s}{[S]} = 1 + \frac{[S]}{K_{ss}}$$

for the competitive case.



(a) (b)

FIG. 1. The relation of the reaction velocity and the pyruvate concentration. (a) is a section of (b), expanded.

A plot of $(v_m/v - K_s/[S])$ against [S] will give a straight line with a slope of $1/K_{ss}$ and an intercept on the y-axis of either unity or of $(1 + K_s/K_{ss})$, depending on whether the inhibition is of the competitive or noncompetitive type. Such a plot is shown in Fig. 2. From the graph it is impossible to determine which type of mechanism is operative, since the value of the fraction K_s/K_{ss}, if involved, would be too small to be detectable by the graphical method. This is evident from the circumstance that the value of K_{ss}, as obtained from the slope of the line plotted in Fig. 2, is 2.4×10^{-3} M.[3] The value of K_s/K_{ss} thus is 1.7×10^{-2}, and gives the intercept on the y-axis a value of 1.017 instead of unity.

[3]This value is in agreement with the value 2.5×10^{-3} M obtained by Hakala *et al.* (3), at 27° C and pH 6.8, conditions which are comparable with ours.

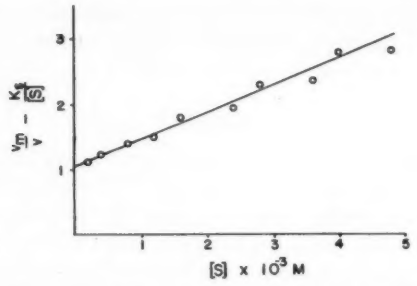


FIG. 2. Inhibition by excess of the substrate. Plot of $(v_m/v - K_s/[S])$ against [S] for the determination of K_{ss} according to equations [25] or [26]. $K_{ss} = 2.4 \times 10^{-3}$ M.

The determination of the mechanism of inhibition of lactic dehydrogenase by high concentrations of its substrate was carried out as follows:

With inhibiting concentrations of pyruvate (4, 8, or 12 $\times$ 10^{-3} M), and with concentrations of oxalate ranging from 4 $\times$ 10^{-5} M to 3 $\times$ 10^{-4} M, the plot of $(v - v_i)$ against $(v - v_i)/[I]$ gives the lines shown in Fig. 3. A subsequent plot of the slopes of these lines against the corresponding substrate concentrations gives the line shown in Fig. 4. The value of the slope is 2.9 $\times$ 10^{-2} and that of the intercept on the y-axis is 7 $\times$ 10^{-5}.

Since these results indicate a dependence of the slopes of the lines shown in Fig. 3, on the concentration of the substrate (pyruvate), one may conclude that, of the three mechanisms specified above, b is the one that was operative.

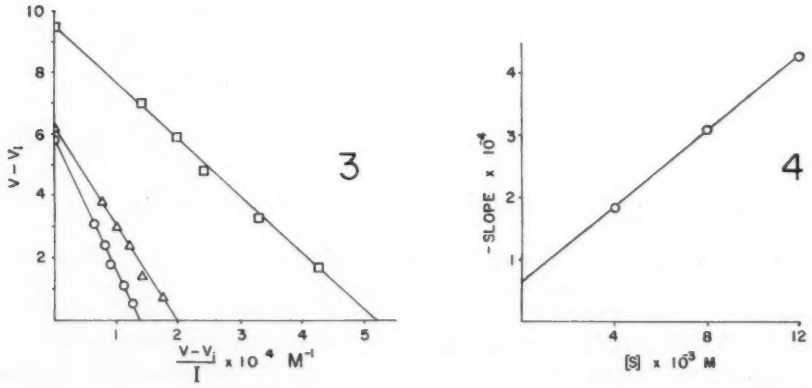


FIG. 3. Simultaneous inhibition by an excess of the substrate and by oxalate. Plot of $(v - v_i)$ against $(v - v_i)/[I]$ (Equation [21] or [23]).
□, 4 $\times$ 10^{-3} M pyruvate.
△, 8 $\times$ 10^{-3} M pyruvate.
○, 1.2 $\times$ 10^{-2} M pyruvate.

FIG. 4. The effect of pyruvate concentration on the slope of the lines of Fig. 3. Plot of the negative slope of the lines in Fig. 3 against the corresponding pyruvate concentration. Slope = 2.9 $\times$ 10^{-2}. Intercept on the y-axis = 7 $\times$ 10^{-5}.

The slope of the plot in Fig. 4 therefore should be equal to K_i/K_{ss}, ($K_i = 7 \times 10^{-5}$ M; $K_{ss} = 2.4 \times 10^{-3}$ M; and, therefore, $K_i/K_{ss} = 2.92 \times 10^{-2}$) and the intercept on the y-axis should equal the K_i (7×10^{-5} M). Both these conditions obviously are fulfilled.

Conclusions

The experimental evidence obtained in this study appears to confirm the postulate that lactic dehydrogenase has two sites, A and B, to which pyruvate, the substrate, can be attached. Only the site A is specific for the activation of the pyruvate molecule. The other site, B, can attach pyruvate but the formation of the complex inactivates the enzyme, that is, it prevents activation of pyruvate at site A. It would appear, furthermore, from the experimental results, that oxalate competes with pyruvate, or vice versa, for occupation of the B site. The inhibition of lactic dehydrogenase by an excess of pyruvate, therefore, is noncompetitive with respect to the inactivation of the A site.

References

1. KUBOWITZ, F. and OTT, P. Biochem. Z. **314**, 94 (1943).
2. NEILANDS, J. B. J. Biol. Chem. **208**, 225 (1954).
3. HAKALA, M. T., GLAID, A. J., and SCHWERT, G. W. J. Biol. Chem. **221**, 191 (1956).
4. HALDANE, J. B. S. Enzymes. Longmans, Green & Co. Ltd., London. 1930. p. 84.
5. LINEWEAVER, H. and BURK, D. J. Am. Chem. Soc. **56**, 658 (1934).
6. KALOW, W., GENEST, K., and STARON, N. Can. J. Biochem. and Physiol. **34**, 637 (1956).
7. HOFSTEE, B. H. J. J. Biol. Chem. **216**, 235 (1955).
8. QUASTEL, J. H. and WOOLDRIDGE, W. R. Biochem. J. **22**, 689 (1928).
9. OTTOLENGHI, P. and DENSTEDT, O. F. (Paper I of this series.) This issue.

LACTIC DEHYDROGENASE OF THE MAMMALIAN ERYTHROCYTE

III. THE FORMATION OF A COMPLEX OF THE ENZYME WITH DPN AND LACTATE[1]

PAUL OTTOLENGHI AND ORVILLE F. DENSTEDT

Abstract

In the transfer of hydrogen ions and electrons from lactate to DPN by lactic dehydrogenase the lactate is capable of forming a complex only with the binary complex of enzyme and coenzyme to form an active ternary complex. The results of kinetic studies indicate that the site for the binding of lactate is on the DPN molecule itself.[2]

The order, if any, in which the substrate and the coenzyme combine with the enzyme is of considerable theoretical interest. In a system in which an enzyme reacts with a substrate and a coenzyme, and in which only the ternary complex breaks down to yield products, the formation of the complex may follow either a definite or an unrestricted order. Three sequences are possible as was shown by Friedenwald and Maengwyn-Davies (1).

Case *a*: when no obligatory order of addition of the substrate and coenzyme to the enzyme is involved. In this case the following equilibria will exist:

[1] $\quad E + S \rightleftharpoons ES \qquad K_s = [E]\,[S] / [ES],$

[2] $\quad E + C \rightleftharpoons EC \qquad K_c = [E]\,[C] / [EC],$

[3] $\quad ES + C \rightleftharpoons ECS \rightarrow P \qquad K_c = [ES]\,[C] / [ECS],$

[4] $\quad EC + S \rightleftharpoons ECS \rightarrow P \qquad K_s = [EC]\,[S] / [ECS].$

In the above equations it is assumed that the combination of the enzyme with one substrate does not affect the subsequent formation of the ternary complex with the second substrate; the equilibrium constants for equations [1] and [4] and for [2] and [3] are therefore the same.

If $E_t = E + ES + EC + ECS$ the concentration of each of the complexes can be expressed in terms of the active complex ECS, and if one makes the assumptions implied in the Michaelis–Menten theories (2) the following relationship will be obtained:

[5] $$\frac{1}{v} = \frac{1}{v_m}\left(1 + \frac{K_s}{[S]}\right)\left(1 + \frac{K_c}{[C]}\right).$$

[1]Manuscript received May 12, 1958.

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[2]The following abbreviations have been used: DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; tris, trishydroxymethylaminomethane; E, free enzyme; S, substrate; C, coenzyme; P, products; ES, EC, ECS, complexes of enzyme with substrate and/or coenzyme; K_c, K_s, dissociation constants; v, velocity of the reaction; v_m, maximum velocity of the reaction.

Double reciprocal (Lineweaver–Burk) plots can be made either of $\frac{1}{v}$ against $\frac{1}{[S]}$ for various concentrations of C, or of $\frac{1}{v}$ against $\frac{1}{[C]}$ for various concentrations of S. The plots in either case give a family of lines with a common intercept on the x-axis. In the plot of $\frac{1}{v}$ against $\frac{1}{[S]}$, the y-intercept for each concentration of C may be represented by $(1 + K_c/[C])/v_m$, the slope by $(1 + K_c/[C])\ K_s/v_m$, and the x-intercept by $-1/K_s$. When the value of [C] is very high compared to K_c the y-intercept will approach the value of $1/v_m$. The slope will then approach the value of K_s/v_m. A plot of $1/v$ against $1/[C]$ is analogous to the above relationships with C substituted for S in each case.

Case *b*: when combination of the coenzyme with the enzyme must occur in order that the substrate can be attached, that is to say, the binding of the coenzyme to the enzyme creates a site for the binding of the substrate. Under these circumstances the following reactions can occur:

[6] $$E + C \rightleftharpoons EC \qquad K_c = [E]\ [C]\ /\ [EC],$$

[7] $$EC + S \rightleftharpoons ECS \rightarrow P \qquad K_s = [EC]\ [S]\ /\ [ECS].$$

In this case $E_t = E + EC + ECS$, from which

[8] $$\frac{1}{v} = \frac{1}{v_m}\left(1 + \frac{K_s}{[S]} + \frac{K_sK_c}{[S][C]}\right).$$

In a plot of $\frac{1}{v}$ against $\frac{1}{[S]}$ for different values of [C], the y-intercepts have a value of $\frac{1}{v_m}$. The slope of each line corresponding to a given concentration of C is represented by $(1 + K_c/[C])\ K_s/v_m$ and the value of the x-intercept is $-[C]/K_s\ (K_c + [C])$. When [C] is very large compared to K_c, the factor $K_c/[C]$ vanishes and equation [8] becomes

[9] $$\frac{1}{v} = \frac{1}{v_m}\left(1 + \frac{K_s}{[S]}\right).$$

In this case the value of the x-intercept is $-1/K_s$.

In a plot of $\frac{1}{v}$ against $\frac{1}{[C]}$ it is evident from equation [8] that the lines for different values of [S] will intersect at a point where coordinates are $(-1/K_c, 1/v_m)$. The y-intercept now can be represented by $(1 + K_s/[S])/v_m$ and the slope of each line by $(K_cK_s/[S])/v_m$. As the value [S] approaches infinity the slope approaches zero and the y-intercept approaches the value of $1/v_m$.

Case *c*: when the coenzyme can combine with the enzyme–substrate complex but cannot combine with the free enzyme. The expressions are the same as in case *b* but with S and C interchanged.

The formation of a complex between lactic dehydrogenase and one form of the coenzyme was indicated by Chance and Neilands (3). With the heart-muscle enzyme they observed a shift in the absorption maximum from 340 to 330 mμ when binding of DPNH to the apoenzyme occurred. Takenaka and Schwert (4) demonstrated the protective action of DPNH towards the inactivation of the enzyme system of heart muscle by *p*-chloromercuribenzoate (*p*CMB) thus indicating the essential role of SH-groups in the binding of the reduced coenzyme. No such direct evidence for the binding of the oxidized coenzyme to the enzyme has been obtained.

From the evidence presented by Schwert *et al.* (5) in an earlier publication with reference to the enzyme of heart muscle, we conclude that in the pyruvate DPNH system the reduced coenzyme and the pyruvate must be attached to different sites on the enzyme. If one uses the data presented by Schwert *et al.* (5) and plots the reciprocal of the reaction velocity, expressed as a function of the reciprocal of the pyruvate concentration for several concentrations of DPNH, one obtains the family of lines shown in Fig. 1. A similar plot is obtained when the reciprocal velocity of the reaction and the reciprocal of the concentration of DPNH are considered as variables for several concentrations of pyruvate (Fig. 2). Such plots indicate that the binding of the substrate to the enzyme does not influence the binding subsequently of the coenzyme, or vice versa. Further, they imply the existence of specific sites on the enzyme surface for the formation of a complex of the substrate and the coenzyme. Thus it is inconsequential for the eventual activation of the system whether the coenzyme or the substrate is the first to become attached. Socquet and Laidler (6) had arrived at a similar conclusion from the interpretation of temperature-coefficient data.

In a more recent publication Hakala, Glaid, and Schwert (7) have presented results which are not interpretable on the basis of a simple mechanism as described above but apparently require the application of the more complex formulations of Alberty (8) for both the lactate–DPN and the pyruvate–DPNH systems. Takenaka and Schwert (4) moreover were unable to find any evidence of the binding of pyruvate or lactate onto the free apoenzyme

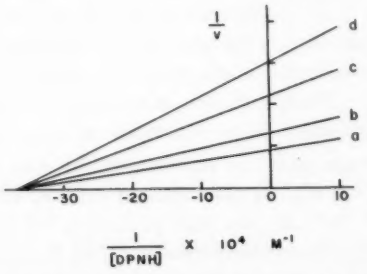

FIG. 1. Effect of the concentration of pyruvate on the reaction velocity and on the Michaelis constant for DPNH. (From Schwert and Hakala (5).) Pyruvate concentrations: (*a*) 2×10^{-4} *M*, (*b*) 5×10^{-4} *M*, (*c*) 2×10^{-3} *M*, (*d*) 1.25×10^{-3} *M*; 5×10^{-2} *M* phosphate buffer, pH 7.3; temperature, 23° C.

when they studied the systems by the ultracentrifugal method of Velick *et al.* (9). Schwert and associates concluded therefore that it is necessary for the apoenzyme to form a complex with the coenzyme before the substrate can be bound to form the active ternary complex.

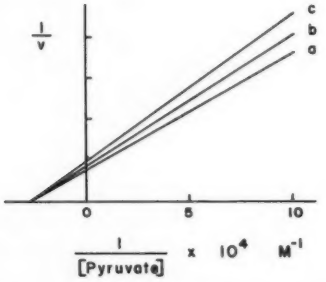

FIG. 2. Effect of the concentration of DPNH on the reaction velocity and on the Michaelis constant for pyruvate. (From Schwert and Hakala (5).) DPNH concentrations: (*a*) 2.32×10^{-4} *M*, (*b*) 2×10^{-5} *M*, (*c*) 1.07×10^{-5} *M*; 5×10^{-2} *M* phosphate buffer, pH 7.3; temperature, 23° C.

Experimental

Methods

The procedures were as described in the first paper of this series (12).

Results and Discussion

With the lactate–DPN system, a plot of the reciprocal of the values of the reaction velocity against the reciprocal of the concentration of lactate for three concentration levels of DPN, gives the lines shown in Fig. 3. A similar treatment of $1/v$ against 1/[DPN] is represented in Fig. 4.

In Fig. 3 it is evident that the lines have a common intercept on the *y*-axis while, in Fig. 4, the common point lies in the second quadrant. These results are consistent with the postulate that a specific order of addition of the coenzyme and substrate to the enzyme is essential. This system corresponds to case *b* discussed above and thus conforms with the conditions implied by equation [8]. Thus a binary complex of the enzyme with DPN is prerequisite to the formation of an activated ternary complex.

In Fig. 4 the common point is represented by the coordinates $(-1/K_c, 1/v_m)$. In this case $-1/K_c$ has the value of 1.74×10^3 M^{-1}. The value of K_c therefore is 5.8×10^{-4} *M*, and that of $1/v_m$ is 9.5×10^{-3} units. By substitution of these values in the expression $(1 + K_c/[C])\ K_s/v_m$ (see case *b*) for the slopes of the lines in Fig. 3 the mean value for the K_s was found to be 8.0×10^{-4} *M*. A similar value is obtained for the K_s on substituting the experimental values into the expression for the *y*-intercepts in Fig. 4.

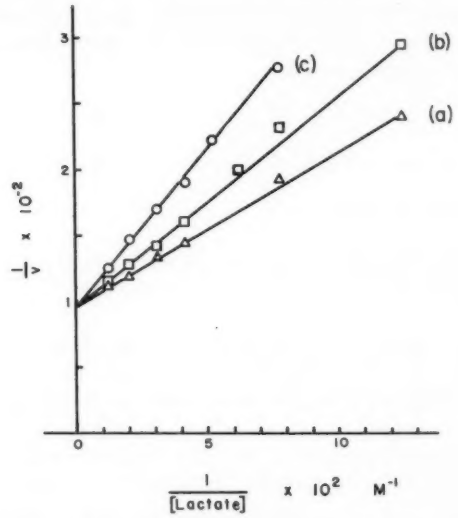

FIG. 3. Effect of change in the concentration of DPN on the Lineweaver–Burk plots of $1/v$ against 1/[lactate]. DPN concentrations: (*a*) 1.08×10^{-3} *M*, (*b*) 5.4×10^{-4} *M*, (*c*) 2.7×10^{-4} *M*; 4×10^{-2} *M* tris buffer, pH 9.2; temperature, 25° C.

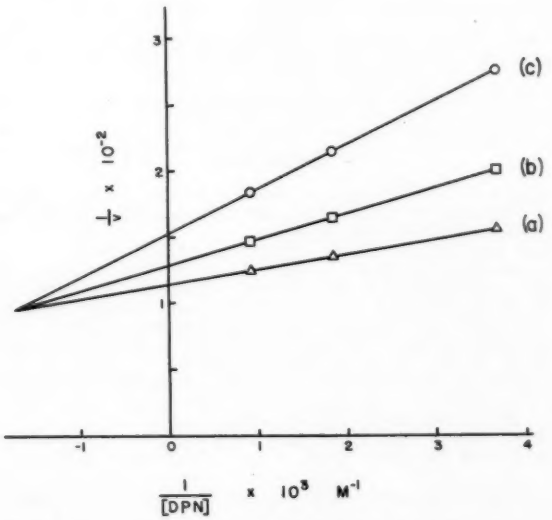

FIG. 4. Effect of change in the lactate concentration on the Lineweaver–Burk plots of $1/v$ against 1/[DPN]. D-Lactate concentrations: (*a*) 4×10^{-3} *M*, (*b*) 2.3×10^{-3} *M*, (*c*) 1.3×10^{-3} *M*; 4×10^{-2} *M* tris buffer, pH 9.2; temperature, 25° C.

From these results one may conclude that the reactions involved in the process of activation of lactate and DPN by lactic dehydrogenase[3] under our experimental conditions, are

$$E + DPN \rightleftharpoons E.DPN \qquad K_e = 5.8 \times 10^{-4}\ M,$$

$$E.DPN + \text{lactate} \rightleftharpoons E.DPN.\text{lactate} \qquad K_s = 8.0 \times 10^{-4}\ M.$$

$$E.DPN.\text{lactate} \rightarrow \text{products}.$$

Although the mechanism represented above is simple compared with that described by Hakala *et al.* (7), the final conclusions as to the reaction sequence are the same.

In view of the required order of addition observed with DPN and lactate it is apparent that the DPN bound to the apoenzyme provides the site for the attachment of lactate and that the lactate becomes bound to the DPN. Since the pyruvate molecule attaches at a site distinct from that at which the lactate molecule is bound[4] (13, 14), and since substrate specificity exists in the DPN-linked dehydrogenases, it is more likely that the pyruvate becomes bound directly to the protein apoenzyme rather than to the pyridine nucleotide. Further evidence for the existence of separate sites for the binding of pyruvate and DPNH has been presented by Winer *et al.* (16). They have shown that oxamate, while inhibiting competitively with respect to pyruvate, is a noncompetitive inhibitor with respect to DPNH. For the activation of the pyruvate–DPNH system it would appear that the order of addition of coenzyme and substrate to the enzyme (5, 6) is unrestricted.

[3]As the Michaelis constant for DPNH with lactic dehydrogenase is very low (7, 10, 11, 12), our methods did not prove sufficiently sensitive to be applied to the study of the pyruvate–DPNH system.

[4]Interaction between these two sites is postulated as will be shown in a further communication (15).

References

1. FRIEDENWALD, J. S. and MAENGWYN-DAVIES, G. D. *In* The mechanism of enzyme action. *Edited by* W. D. McElroy and B. Glass. The Johns Hopkins University Press, Baltimore, Md. 1954. p. 154.
2. MICHAELIS, L. and MENTEN, M. L. Biochem. Z. **49**, 333 (1913.
3. CHANCE, B. and NEILANDS, J. B. J. Biol. Chem. **199**, 383 (1952).
4. TAKENAKA, Y. and SCHWERT, G. W. J. Biol. Chem. **223**, 157 (1956).
5. SCHWERT, G. W. and HAKALA, M. T. Arch. Biochem. Biophys. **38**, 55 (1952).
6. SOCQUET, I. M. and LAIDLER, K. J. Arch. Biochem. **25**, 171 (1950).
7. HAKALA, M. T., GLAID, A. J., and SCHWERT, G. W. J. Biol. Chem. **221**, 191 (1956).
8. ALBERTY, R. A. J. Am. Chem. Soc. **75**, 1928 (1953).
9. VELICK, S., HAYES, J., and HARTING, J. J. Biol. Chem. **203**, 527 (1953).
10. KUBOWITZ, F. and OTT, P. Biochem. Z. **314**, 94 (1943).
11. NEILANDS, J. B. J. Biol. Chem. **199**, 373 (1952).
12. NYGAARD, A. P. Acta Chem. Scand. **10**, 408 (1956).
13. OTTOLENGHI, P. and DENSTEDT, O. F. Can. J. Biochem. and Physiol. Paper I. This issue.
14. OTTOLENGHI, P. and DENSTEDT, O. F. Can. J. Biochem. and Physiol. Paper II. This issue.
15. OTTOLENGHI, P. and DENSTEDT, O. F. In preparation.
16. WINER, A. D., NOVOA, W. B., and SCHWERT, G. W. J. Am. Chem. Soc. **79**, 6571 (1957).

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